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Larvicidal activity of green synthesized silver nanoparticles using bark aqueous extract of *Ficus racemosa* against *Culex quinquefasciatus* and *Culex gelidus*

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ABSTRACT

Objective: To investigate the larvicidal activity of synthesized silver nanoparticles (Ag NPs) utilizing aqueous bark extract of *Ficus racemosa* (*F. racemosa*) was tested against fourth instar larvae of filariasis vector, *Culex quinquefasciatus* (*Cx. quinquefasciatus*) and japanese encephalitis vectors, *Culex gelidus* (*Cx. gelidus*). **Methods:** The synthesized Ag NPs was characterized by UV-vis spectrum, X-ray diffraction (XRD), Scanning electron microscopy (SEM) and Fourier transform infrared (FTIR). The larvicidal activities were assessed for 24 h against the larvae of *Cx. quinquefasciatus* and *Cx. gelidus* with varying concentrations of aqueous bark extract of *F. racemosa* and synthesized Ag NPs. LC_{50} and r^2 values were calculated. **Results:** The maximum efficacy was observed in crude aqueous extract of *F. racemosa* against the larvae of *Cx. quinquefasciatus* and *Cx. gelidus* (LC_{50}=67.72 and 63.70 mg/L; r^2=0.995 and 0.985) and the synthesized Ag NPs (LC_{50}=12.00 and 11.21 mg/L; r^2=0.997 and 0.990), respectively. Synthesized Ag NPs showed the XRD peaks at 2θ values of 27.61, 29.60, 35.48, 43.48 and 79.68 were identified as (210), (121), (220), (200) and (311) reflections, respectively. The FTIR spectra of Ag NPs exhibited prominent peaks at 3 425, 2 878, 1 627 and 1 382 in the region 500-3 000 cm^{-1}. The peaks correspond to the presence of a stretching vibration of (NH) C=O group. SEM analysis showed shape in cylindrical, uniform and rod with the average size of 250.60 nm. **Conclusions:** The biosynthesis of silver nanoparticles using bark aqueous extract of *F. racemosa* and its larvicidal activity against the larvae of disease spreading vectors. The maximum larvicidal efficacy was observed in the synthesized Ag NPs.

1. Introduction

Mosquitoes are important vectors of diseases, especially in the tropics. Regulation of mosquito populations to reduce the incidence of disease like malaria, filariasis and several arboviruses are importance from public health viewpoint. Owing to the problems associated with resistance and effects on non-target species by chemicals[1]. Filariasis is endemic in 17 States and six Union Territories, with about 553 million people at risk of infection[2]. However, chronic manifestations, such as lymphedema (elephantiasis) and hydrocele are debilitating and estimated by the World Health Organization to account for nearly five million disability adjusted life years[3]. Japanese encephalitis is the most important cause of viral encephalitis in Eastern and Southeast Asia. Up to 50 000 cases and 15 000 deaths annually are due to JE especially in the rural areas[4,5].

The target species vector control is facing a threat due

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to the development of resistance to chemical insecticides resulting in rebounding vectorial capacity[6]. Insecticides have provoked undesirable effects, including toxicity to non-target organisms and fostered environmental and human health concerns[7]. The Ag NPs which are less likely to cause ecological damage have been identified as potential replacement of synthetic chemical insecticides, hence the need to use green synthesized Ag NPs for the control of disease vectors.

The Ag NPs may be released into the environment from discharges at the point of production, from erosion of engineered materials in household products (antibacterial coatings and silver-impregnated water filters) and from washing or disposal of silver containing products[8]. Silver has been known to exhibit strong toxicity to a wide range of microorganisms and has been used extensively in many antibacterial applications[9]. The green synthesis of Ag NPs by various plants has been reported, the potential of plants as biological materials for the synthesis of nanoparticles are yet to be fully explored[10]. Recent reports include the biosynthesis of Ag NPs using leaf extracts of *Manilkara zapota (M. zapota)*[11], *Mimosa pudica (M. pudica)*[12] and fruit peel extract of *Musa paradisiaca (M. paradisiaca)*[13] against *Rhipicephalus microplus (R. microplus)*, the fourth-instar larvae of *Anopheles subpictus* (*An. subpictus*), *C. quinquefasciatus*, *Anopheles stephensi (An. stephensi)*, and *Culex tritaeniorhynchus (Cx. tritaeniorhynchus)*.

Ficus racemosa (*F. racemosa*) L.(Moraceae) has been used in Indian folk medicine for the treatment of various diseases/disorders including jaundice, dysentery, diabetes, diarrhea and inflammatory conditions[14]. The compound of racemosic acid, gluanol acetate, caoutchouc, tannins, β-sitosterol, stigmasterol, friedelin and hentriacontane from the bark of *F. racemosa*[15]. The *F. racemosa* bark showed hepatoprotective, chemopreventive, anti-diabetic, anti-inflammatory, anti-pyretic, anti-tussive, and anti-diuretic effects[16]. The crude aqueous extract of the latex of *Ficus benghalensis (F. benghalensis)* was tested against the fourth instar larvae of *C. quinquefasciatus*[17]. The insecticidal efficacy of different concentrations of fruit pericarp methanol extract of *Artocarpus lakoocha (A. lakoocha)*(Moraceae) was evaluated against second and third instar larvae of *Aedes aegypti (Ae. aegypti)*[18].

The use of plants for synthesize of nanoparticles are rapid low cost, eco-friendly and safe for human therapeutic use[19]. Evaluation of synthesized Ag NPs using leaf aqueous extract of *Lawsonia inermis (L. inermis)* used to control *Pediculus humanus capitis (P. h. capitis)* and *Bovicola ovis (B. ovis)* [20]. Nair *et al*[21] reported that the Ag NPs did not have acute toxicity against the fourth instar larvae of the aquatic midge *Chironomus riparius (C. riparius)*, but exhibited chronic toxicity on the development (pupation and emergence failure) and reproduction. A comparative assessment of the 48 h acute toxicity of synthesized Au, Ag, and Ag-Au bimetallic nanoparticles was conducted to determine their ecological effect in freshwater environments through the use of *Daphnia magna (D. magna)*[22]. The current study aimed to explore the larvicidal activity of green synthesized Ag NPs using aqueous bark extract of *F. racemosa* to control *C. quinquefasciatus* and *C. gelidus*.

2. Materials and methods

2.1. Preparation of aqueous bark extract of F. racemosa

F. racemosa bark was collected from Melvisharam, Tamil Nadu, India. The bark was washed thoroughly to remove impurities and under shade dried for about three weeks to remove the moisture. The bark was cut into small pieces, powdered in a mixer and then sieved using 20 mesh size sieves to get uniform size range. Aqueous extract was prepared by mixing 50 g of dried leaf powder with 500 mL of water (boiled and cooled distilled water) with constant stirring on a magnetic stirrer[23]. The suspension of dried bark powder in water was left for 3 h, filtered through Whatman no. 1 filter paper, and the filtrate was stored in amber colored air tight bottle at 10 ℃ and used within a week.

2.2. Synthesis of Ag NPs by F. racemosa bark extract

For the production of aqueous extract, 2.5 g of *F. racemosa* bark powder was added to a 100 mL Erlenmeyer flask with 250 mL sterile distilled water and then boiled for 5 min. The extract was filtered with Whatman filter paper No. 1. The filtrate was treated with aqueous 1 mM silver nitrate ($AgNO_3$) solution in an Erlenmeyer flask and incubated at room temperature. 80 mL aqueous solution of 1 mM of $AgNO_3$ was reduced using 20 mL of bark extract at room temperature for 10 min, resulting in a brown solution indicating the formation of Ag NPs[24].

2.3. Insect rearing

Cx. quinquefasciatus and *Cx. gelidus* larvae were collected from stagnant water area of Melvisharam (12°56 23″N, 79° 14′23″ E) and identified in Zonal Entomological Research Centre, Vellore (12°55′48″ N, 79°7′48″ E), Tamil Nadu. To start the colony, the larvae were kept in plastic and enamel trays containing tap water. They were maintained and reared in the laboratory as per the method[25]. The larvae of *Cx. quinquefasciatus* and *Cx. gelidus* were collected from the insect rearing cage and identified in Zonal Entomological Research Centre, Vellore. One gram of aqueous leaf extract was first dissolved in 100 mL of distilled water for bioassay test of plant extract (stock solution). The larvicidal activity was assessed by the procedure of WHO[26] with some modification and as per the method of Rahuman *et al*[27]. For the bioassay test, larvae were taken in five batches of 20 in 249 mL of water and 1.0 mL of the desired plant extract concentration. Control was set up with dechlorinated tap water. The numbers of dead larvae were counted after 24 h of exposure, and the percent mortality was reported from the average of five replicates. The experimental media, in which 100% mortality of larvae occurs alone, were selected for dose response bioassay.

Synthesized Ag NPs toxicity test was performed by placing 20 mosquito larvae into 200 mL of sterilized double distilled water with Ag NPs in a 250 mL beaker (Borosil). 100 mg of synthesized Ag NPs was first dissolved in 1 L of Milli Q water (stock solution). From the stock solution, the nanoparticle solutions were diluted using Milli Q water as a solvent according to the desired concentrations (5, 10, 15, 20 and 25 mg/L). Each test included a set control group (distilled water) with five replicates for each individual concentration. Mortality was assessed after 24 h to determine the acute toxicities on fourth instar larvae of *Cx. quinquefasciatus* and *Cx. gelidus*. To avoid settling of particles especially at higher doses, all treatment solutions were sonicated for an additional of 5 min prior to addition of the mosquito larvae.

2.4. Dose-response bioassay

During the laboratory trial, the crude bark extract of *F. racemosa* and synthesized Ag NPs were subjected to a dose-response bioassay for larvicidal activity against *Cx. quinquefasciatus* and *Cx. gelidus*. Different concentrations ranging from 20, 40, 60, 80 and 100 mg/L (for aqueous plant extracts) and 5, 10, 15, 20, and 25 mg/L (for synthesized Ag NPs) were prepared for larvicidal activity. The numbers of dead larvae were counted after 24 h of exposure, and the percent mortality was reported from the average of five replicates. However, at the end of 24 h, the selected test samples turned out to be equal in their toxic potential.

2.5. Characterization of the synthesized nanoparticles

Synthesis of Ag NPs solution with bark extract was observed by UV-vis spectroscopy. The bioreduction of the Ag^+ ions in solutions was monitored by periodic sampling of aliquots (1 mL) of the aqueous component after 20 times dilution and measuring the UV-vis spectra of the solution. UV-vis spectra of these aliquots were monitored as a function of time of reaction on a Schimadzu 1601 spectrophotometer in 300-700 nm range operated at a resolution of 1 nm. Further, the reaction mixture was subjected to centrifugation at 5 000 rpm for 30 min; resulting pellet was dissolved in deionized water and filtered through Millipore filter (0.45 μm). Fourier transform infrared (FTIR) spectra of the samples were measured using a Perkin Elmer Spectrum One instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets. Powder samples for the FTIR was prepared similarly as for powder diffraction measurements. The FTIR spectra of bark extracts taken before and after synthesis of Ag NPs were analyzed which discussed for the possible functional groups for the formation of Ag NPs. An aliquot of this filtrate containing Ag NPs was used for X-ray diffraction (XRD) and FTIR analysis. For XRD studies, dried nanoparticles were coated on the XRD grid, and the spectra were recorded using Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with CuK α 1 radiation. For scanning electron microscopy studies, 25 μL of sample was sputter-coated on copper stub, and the images of nanoparticles (SEM; JEOL, Model JFC-1600).

2.6. Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating LC_{50} and other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit were calculated by using the software developed by Reddy *et al*[28]. Results with $P<0.05$ were considered to be statistically significant.

3. Results

In the present study, the larvicidal aqueous crude bark extracts and synthesized Ag NPs of *F. racemosa* were noted; however, the highest mortality was found in synthesized Ag NPs against the larvae of *Cx. quinquefasciatus* and *Cx. gelidus* at the concentration of 25 mg/L. The larvicidal activity of aqueous crude bark extracts and synthesized Ag NPs of *F. racemosa* showed the LC_{50} (UCL-LCL) values of 67.72 (61.5-74.74) and 12.00 (9.3-13.01) mg/L; r^2 values of 0.995 and 0.997 against *Cx. quinquefasciatus* and 63.70 (57.3-70.88) and 11.21(10.0-14.09) mg/L; r^2=0.985 and 0.990 against *Cx. gelidus*, respectively (Figure 1). The larvicidal activity results showed the highest mortality in synthesized Ag NPs than the aqueous bark extract of *F. racemosa*. All the tested components that showed lethal effect and mortality were positively dose-dependent. The results showed that the optimal hours for measuring the percent mortality in aqueous bark extract and synthesized Ag NPs against were 9, 26, 39, 57, 77 and 24, 42, 58, 79 and 100 against *Cx. quinquefasciatus* and 13, 27, 48, 60, 72 and 28, 39, 64, 82 and 100 against *Cx. gelidus* at 1, 6, 12, 18 and 24 h, respectively (Figure 2).

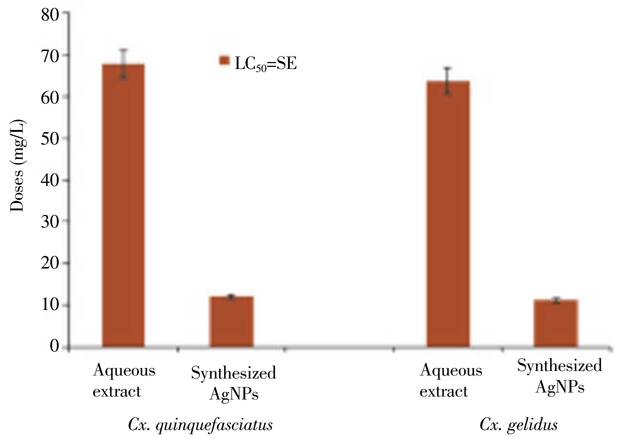

Figure 1. Graph showing the LC_{50} values of *Cx. quinquefasciatus* and *Cx. gelidus* larvae.

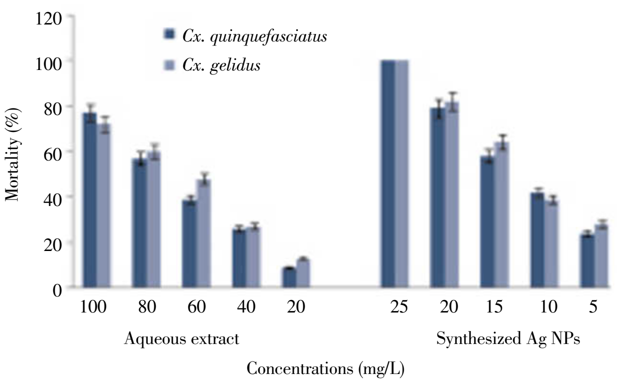

Figure 2. Graph showing the larvicidal activity of aqueous extract of *F. racemosa* and synthesized Ag NPs against fourth instar larvae of *Cx. quinquefasciatus* and *Cx. gelidus*.

The pure $AgNO_3$ without aqueous bark extract of *F. racemosa* didn't show any colour change and there was no proof for the formation of Ag NPs. Ag NPs were synthesized rapidly within 30 minutes of incubation period. The aqueous silver nitrate solution was turned to brown color within 30mins, with the addition of bark extract. Intensity of brown color increased in direct proportion to the incubation period. Absorption spectrum of synthesized Ag NPs with bark aqueous extract of *F. racemosa* at different wave lengths ranging from 300 to 600 nm revealed a peak at 425 nm (Figure 3). The XRD patterns of vacuum dried Ag NPs synthesized using bark extract of *F. racemosa*. A number of Bragg reflections with 2 θ values of 27.61, 29.60, 35.48, 43.48 and 79.68 sets of lattice planes were observed and indexed to (210), (121), (220), (200) and (311) facts of silver, respectively (Figure 4). The XRD results also suggest that crystallization of the bioorganic phase occurs on the surface of the Ag NPs. The FTIR band intensities in different regions of the spectrum for the *F. racemosa* bark powder and synthesized Ag NPs test samples were analyzed. There was a shift in the following peak and the spectra showed sharp and strong absorption band at 1 620 to 1 627 cm^{-1} assigned to the stretching vibration of (NH) C=O group. The band 1 373 to 1 382 cm^{-1} developed for C–C and C–N stretching, respectively and was commonly found in the proteins. The presence of the sharp peak at 2 922 to 2 878 cm^{-1} was assigned to C–H and C–H (methoxy compounds) stretching vibration, respectively (Figure 5 A and B). The SEM micrograph shows the synthesized nanoparticles were cylindrical, uniform, rod shaped and with an average size of 250.60 nm (Figure 6A, B and C).

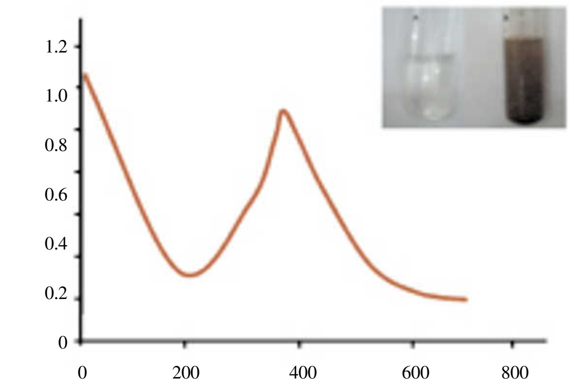

Figure 3. UV–vis spectra of silver nanoparticles synthesized using aqueous bark extracts of *F. racemosa*.

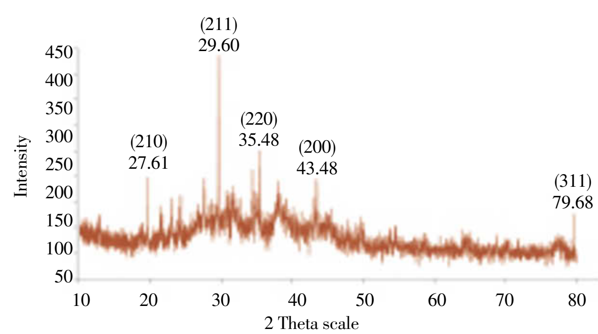

Figure 4. XRD pattern of silver nanoparticles synthesized using aqueous bark extracts of *F. racemosa*.

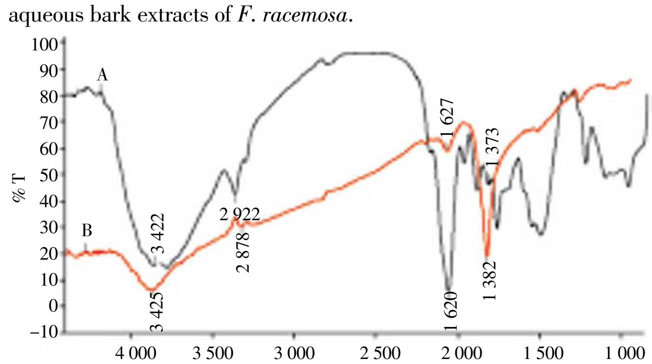

Figure 5. FTIR spectrum of (A) bark powder of *F. racemosa* (B) synthesized silver nanoparticles using aqueous bark extracts of *F. racemosa*.

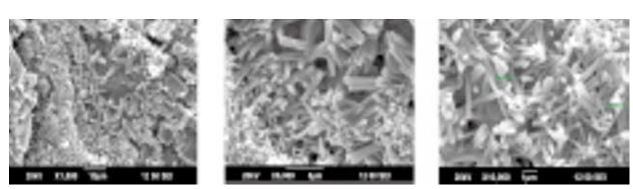

Figure 6. SEM micrograph A) ×1 500 10 μ m; B) ×5 000 5 μ m; C) ×10 000 1 μ m showing the silver nanoparticles synthesized using bark aqueous extract of *F. racemosa*.

4. Discussion

In the present study, the larvicidal activity of aqueous bark extracts and synthesized Ag NPs of *F. racemosa* was noted. However, the activity was observed in aqueous bark extract of *F. racemosa* and the synthesized Ag NPs against *Cx. quinquefasciatus* and *Cx. gelidus*. Rahuman *et al*[29] have reported that the bioassay-guided fractionation of acetone extract of *F. racemosa* led to the separation and identification of a tetracyclic triterpenes derivative; gluanol acetate was isolated and identified as a new mosquito larvicidal compound and it was quite potent against fourth instar larvae of *Ae. aegypti* (LC_{50} =14.55 and LC_{90} = 64.99 ppm), *An. stephensi* (LC_{50} =28.50 and LC_{90} =106.50 ppm) and *Cx. quinquefasciatus* (LC_{50}=41.42 and LC_{90}=192.77 ppm). The maximum efficacy was observed in methanol extract with the lethal concentration (LC_{50}) values of *F. benghalensis* against early second, third and fourth larvae of *Cx. quinquefasciatus* were 41.43, 58.21 and 74.32 ppm, respectively[30]. The milky sap of *Ficus carica* have a significant toxic effect against early fourth stage larvae of *Ae. aegypti* with an LC_{50} value of 10.2 μg/mL and an LC_{90} value of 42.3 μg/mL[31].

Madhumitha *et al*[32] have reported the larval parasitic mortality observed in fruit peel aqueous extract of *Annona squamosa (A. squamosa)* were 36%, 55%, 72%, 92%, 100% and 14%, 34%, 68%, 89%, and 100% at 200, 400, 600, 800, and 1 000 ppm, respectively, against *An. subpictus* and *Cx. quinquefasciatus* and the highest parasite mortality was found after 24 h of exposure against fourth instar larvae of *An. subpictus* (LC_{50} = 327.27 ppm, r^2=0.970), *Cx. quinquefasciatus* (LC_{50}=456.29 ppm, r^2=0.974), respectively. The larvicidal effects of aqueous extracts from leaves of *Ricinus communis (R. communis)* showed the LC_{50} values of 1 091.44, 1 364.58 and 1 445.44 ppm against 2nd, 3rd and 4th larval instars of *Cx. quinquefasciatus*[33]. 55% mortality was observed in 2.5% concentration of aqueous extract of dried leaves of *Caesalpinia bonduc (C. bonduc)* tested against the fourth instar larvae of *Cx. quinquefasciatus*[34].

Aqueous extracts of *Azadirachta indica (A.indica)*, *Gymnema sylvestre (G. sylvestre)*, *Nerium indicum (N. indicum)* and *Datura metel (D. metel)* were tested in a laboratory for larvicidal properties against *Cx. quinquefasciatus* and the results of *A. indica* seeds showed high toxicity with LC_{50} value of 0.53 ppm and LC_{90} value of 3.42 ppm; *G. sylvestre and N. indicum* also showed the LC_{50} values less than 2.00 ppm, while *D. metel* showed 3.97 ppm value[35]. The effects of the aqueous extracts of whole plants of *Striga hermonthica (S. hermonthica)* and *Mitracarpus scaber (M. scaber)* against the larvae of *Cx. quinquefasciatus* were investigated and showed 100% mortality was observed at 1% and 0.5% of *S. hermonthica* and *M. scaber*, respectively[36-40].

The maximum larvicidal activity was observed in the synthesized Ag NPs using leaf aqueous extract of *Tinospora cordifolia (T. cordifolia)* against fourth instar larvae of *An. subpictus* and *Cx. quinquefasciatus* (LC_{50}=6.43 and 6.96 mg/L; r^2=0.773 and 0.828), respectively[41]. Santhoshkumar *et al*[42] reported the maximum efficacy was observed in crude methanol, aqueous, and synthesized Ag NPs using aqueous leaf extract of *Nelumbo nucifera (N.nucifera)* against the larvae of *An. subpictus* (LC_{50}=8.89, 11.82, and 0.69 ppm) and against the larvae of *Cx. quinquefasciatus* (LC_{50}=9.51, 13.65, and 1.10 ppm), respectively. The larvicidal activity of synthesized Ag NPs utilizing aqueous extract from *Eclipta prostrata (E. prostrata)* was investigated and the maximum efficacy was observed in crude aqueous, and synthesized Ag NPs against fourth instar larvae of *Cx. quinquefasciatus* (LC_{50}=27.49 and 4.56 mg/L; LC_{90}=70.38 and 13.14 mg/L), and against *An. subpictus* (LC_{50}=27.85 and 5.14 mg/L; LC_{90}=71.45 and 25.68 mg/L), respectively[43]. The LC_{50} values for second and fourth larval instars after 24 h of synthesized Ag NPs using *Plumeria rubra (P. rubrum)* latex exposure were 1.49, 1.82 ppm against *Ae. aegypt*i and 1.10, 1.74 ppm against *An. stephensi,* respectively and the crude aqueous latex of *P. rubrum* were 181.67, 287.49 ppm against *Ae. aegypti* and 143.69, 170.58 ppm against *An. stephensi*, respectively[44]. The median lethal concentrations (LC_{50}) of synthesized stable silver nanoparticles using *A. squamosa* leaf broth that killed fourth instar larvae of *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi* were 0.30, 0.41, and 2.12 ppm, respectively[45]. Fungus mediated synthesis of Ag NPs using *Chrysosporium tropicum (C. tropicum)* showed efficacy (LC_{50}=3.47, 4, and 2; LC_{90}=12.30, 8.91, and 4; LC_{99}=13.18, 13.18, and 7.58, respectively) after 1 h against the second instar larvae of *Ae. aegypti*[46]. The Ag NPs synthesized by filamentous fungus *Cochliobolus lunatus (C. lunatus)* and the efficacy tested concentrations (10, 5, 2.5, 1.25, 0.625, and 0.3125 ppm) against second, third, and fourth instar larvae of *Ae. aegypti* (LC_{50}=1.29, 1.48, and 1.58; LC_{90}=3.08, 3.33, and 3.41 ppm) and against *An. stephensi* (LC_{50}=1.17, 1.30, and 1.41; LC_{90} =2.99, 3.13, and 3.29 ppm) were observed, respectively[47].

The XRD pattern of pure silver ions was known to display peaks at 2θ = 7.9°, 11.4°, 17.8°, 30.38° and 44°[48]. Jayaseelan and Rahuman[49] reported that the XRD patterns of vacuum dried Ag NPs synthesized using the leaf extract of *Ocimum canum (O. canum)* and the number of Bragg reflections with 2θ values of 27.74° (210), 32.15° (122) and 36.19° (128). XRD pattern of Ag NPs after reaction showed the diffraction peaks at 2θ =38.28°, 46.40°, 64.21° and 77.78° assigned to the (111), (200), (220) and (311) planes of a faced center cubic lattice of silver[50]. Therefore XRD results also suggest that crystallization of the bioorganic phase occurs on the surface of the Ag NPs.

FTIR spectrum the most intense band at 1 620-1 636 cm^{-1} represent carbonyl groups from polyphenols such as catechin gallate, epicatechin gallate, epigallocatechin, epigallocatechin gallate, gallocatechin gallate and the aflavin; the results suggest that molecules attached with Ag NPs have free and bound amide groups. These amide groups may also be in the aromatic rings. This concludes that the compounds attached with the Ag NPs could be polyphenols with an aromatic ring and bound amide region[51]. The peak at 1 381 cm^{-1} corresponds to the C-N stretching of the aromatic amine group[52]. This suggests the attachment of some polyphenolic components onto Ag NPs. This means that polyphenols attached to Ag NPs may have at least one aromatic ring. SEM image, the size of the control silver nitrate obtained was more than 1 000 nm, whereas synthesized Ag NPs measured 25–150 nm in size[53].

Mechanisms of toxicity are still poorly understood although it seems clear that in some cases, nanoscale specific properties may cause bio–uptake and toxicity over and above that caused by the dissolved Ag ion[54]. The exact mechanism of the formation of these nanoparticles in these biological media is unknown. Presumably biosynthetic products or reduced cofactors play an important role in the reduction of respective salts to nanoparticles. It seems quite probable that the phenols play an important part in the reduction of ions to Ag NPs as the concept of antioxidant action of phenol compounds is not new.

The present green synthesis shows that the environmentally benign and renewable source of *F. racemosa* used as an effective reducing agent for the synthesis of Ag NPs. This biological reduction of metal would be boon for the development of clean, nontoxic and environmentally acceptable "green approach" to produce metal nanoparticles, involving organisms even ranging higher plants.

Conflict of interest statement

We declare that we have no conflict of interest.

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