Indian Journal of Geo Marine Sciences
Vol. 49 (12), December 2020, pp. 1831-1838

Assessment of the effect of green synthesized silver nanoparticles against aquatic pathogen *Aeromonas hydrophila* using *Artemia* nauplii

K Madhuri[a], D Divya[a], N M Vinita[b], E Kannapiran*[,c], B Malaikozhundan[d] & R Kumar[a]

[a]Aquatic Microbiology Lab, Department of Animal Health and Management, Alagappa University, Karaikudi, Tamil Nadu – 630 003, India

[b]Molecular Cancer Biology Lab, Department of Animal Health and Management, Alagappa University, Karaikudi, Tamil Nadu – 630 003, India

[c]Department of Fisheries Science, Alagappa University, Karaikudi, Tamil Nadu – 630 003, India

[d]Department of Biology, Gandhigram Rural Institute (Deemed to be University), Gandhigram, Dindigul District, Tamil Nadu – 624 302, India

*[E-mail: ekannapiran@gmail.com]

Received 03 December 2019; 26 February 2020

Fish disease is a major threat affecting the long-term development of aquaculture industry leading to great economic loss annually. *Aeromonas hydrophila* bacterium may be a primary or secondary cause of ulcers, hemorrhagic septicemia, fin rot and tail rot in fishes. Excess and abandoned usage of antibiotic substances caused a substantial growth of antibiotic resistant pathogens. Now a days, enduring research has focused on improvement of nanomaterials for efficient antimicrobial therapies. Comparing to the synthesis of other metallic nanoparticles, silver nanoparticles (AgNPs) has gained much attention because of its unique antimicrobial properties. The present study was aimed to green synthesize AgNPs using the *Annona squamosa* leaf extract which acts as a non-toxic reducing agent. The synthesized AgNPs were evaluated for biocompatibility and antagonistic efficacy against *A. hydrophila*. The green synthesized AgNPs were bio-physically characterized by UV-Visible spectroscopy, X-ray powder diffraction, High Resolution Transmission Electron Microscope, Fourier-transform infrared spectroscopy and Zeta Potential analysis. The particles were almost spherical shaped and in the range of 15-25 nm. Green synthesized AgNPs exhibited significant DPPH radical scavenging activity (64.62 %). Synthesis of AgNPs using widely available plants can be promoted as a potential eco-friendly option to chemical methods used for nanosynthesis. *In-vivo* studies confirmed that the AgNPs exhibited good antagonistic against *A. hydrophila* and also proved its non-toxic effect to *Artemia salina* nauplii.

[**Keywords**: *Aeromonas hydrophila*, *Annona squamosa*, Antibacterial, *Artemia* nauplii, Biocompatibility, Silver nanoparticles]

Introduction

Currently, aquaculture production sector has the highest growth in fisheries. Fishery creates inexhaustible financial gain than any other farming activities. This sector is prone to several challenges together with overexploitation, pollution, climate change leading to diseases with stunted growth causing extensive financial losses. Among aquatic pathogens, *Aeromonas hydrophila* causes outbreak of various fish diseases like ulcers, hemorrhagic septicemia, fin-rot, tail-rot, etc.[1]. To maintain sustainable production, health management practices identify anticipated disease and their cure. Stressful conditions and immune suppression provide a favorable environment for *A. hydrophila*[2]. To prevent disease outbreaks, antibiotics are commonly used in aquaculture. Recent reports revealed that *A. hydrophila* had developed resistance to numerous antibiotics including amoxicillin, penicillin, ampicillin, oxacillin, and tetracycline[3]. Therefore, attempts to find alternatives antibiotic compounds are increasing[4].

In recent years, nanotechnology has gained much interest in biology[5]. It is possible to produce variety of NPs by controlling the shape and size[6]. Unique properties of metal nanoparticles have attracted the attention of researchers. Metal nanoparticles possess applications in various fields such as medicine, environment clean-up, electricals and in electronics. Unusual properties of nanoparticles depend on their small size, chemical composition, surface coating etc.[7]. Different methods such as chemical, physical, and biological methods are in practice for nanoparticle production. However, the chemical methods of nanoparticle synthesis are toxic to the environment

due to the generation of hazardous byproducts[8]. Hence, a safe and eco-friendly procedure is needed for nanoparticle synthesis. The biological methods of nanoparticle synthesis are relatively safer to the environment and are inexpensive. In the biosynthesis of nanoparticles, the reducing agent and the stabilizers are been replaced by the biomolecules. Biological synthesis using microorganisms, seaweeds[9], marine sponges[10] etc. have been suggested for the biosynthesis of AgNPs. However, biological synthesis of AgNPs using microorganisms is not economical and feasible for large scale production due to the requirements of strict sterile environment, facilities for their maintenance and possible bio-hazardous nature. Recently, biosynthesis of nanoparticles using plant extracts has gained much interest as these are highly advantageous over other methods because of the toxic free chemical constituents and due to exclusion of need for cell culture[11,12]. A variety of plants including *Piper pedicellatum*[13], *Myrmecodia pendans*[14], *Desmodium gangeticum*[15], *Syzygium cumini*[16], *Boswellia serrata*[17] etc. were tested for the synthesis of silver nanoparticles. The present study was aimed to assess the antagonistic, antioxidant activity of green synthesized AgNPs from *Annona squamosa* leaf extract and evaluation of their biocompatibility in *Artemia* nauplii.

Materials and Methods

Chemicals and materials

Silver nitrate ($AgNO_3$) and DPPH (2, 2-diphenyl-1-picrylhydrazyl) were procured from Sisco Research Laboratory, India. Bacterial media was purchased from Himedia Pvt. Ltd, Mumbai, India. Fresh leaves of *A. squamosa* were collected in the locality of Karaikudi, Tamil Nadu, India and the specimen was authenticated by Dr. Ramalingam Kottaimuthu, Department of Botany, Alagappa University, Karaikudi, India.

Green synthesis of silver nanoparticles

Fresh leaves of *A. squamosa* (10 g) were washed with distilled water to remove dust. Small pieces of leaves were boiled in 100 ml distilled water for 5 min. Thereafter, the extract was filtered through Whatman No.1 filter paper. Aqueous leaf extract was mixed with 10 parts of $AgNO_3$ (1 mM) and kept stirred at room temperature. The bio-reduction of $AgNO_3$ was monitored by UV–Visible spectra at different time intervals.

Characterization of nanoparticles

UV-Visible spectroscopic analysis of AgNPs was measured at 300 to 700 nm. XRD analysis of AgNPs was determined using Philips PW1830 X-Ray Diffractometer. The particle morphology was observed using JEOL JEM 2100 High Resolution Transmission Electron Microscope (HRTEM). The surface charge of nanoparticles was identified by using Zetasizer Nano S90-Malvern Instrument. FTIR spectrum was also recorded to identify the bio-functional group on the surface of nanoparticles using Thermo Scientific Nicolet iS5 FTIR Spectrometer.

In vitro antioxidant assay

In vitro antioxidant activities of AgNPs such as DPPH radical scavenging activity was evaluated using L-ascorbic acid as standard. Briefly, 4.0 ml of different concentrations of the AgNPs were mixed with 1.0 ml of DPPH solution to get 0.2 mM of DPPH. The solution was mixed thoroughly and allowed to stand for 30 min. The absorbance of the resultant solution was measured at 517 nm and the percentage of inhibition in DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = [(Abs_{control} - Abs_{sample}) / (Abs_{control}) \times 100$$

In vitro antibacterial assessment

The aquatic bacterial pathogen *A. hydrophila* was obtained from the Microbial Type Culture Collection, Indian Institute of Microbial Technology, Chandigarh, India. Antibacterial activity of synthesized AgNPs was evaluated by agar well diffusion method. Pure cultures of *A. hydrophila* with a cell density of 1×10^5 cells/ml were swabbed onto Mueller Hinton agar. Wells of 6 mm diameter were prepared with the help of a sterile cork borer. Various concentrations of AgNPs (0, 25, 50, 75 and 100 µg/ml) were added into the respective pre-labeled wells and the plates were incubated at 35 °C for 24 h.

In vivo study using *Artemia* as model

In vivo experiments were performed with *Artemia salina* cysts (San Francisco Bay Brand, San Francisco, CA, USA). *Artemia* nauplii were hatched by addition of 100 mg of de-capsulated cysts in 100 ml of sterile seawater (salinity of 30 ppt) and were aerated for 24 h[18,19]. Healthy nauplii were allocated into 4 groups with each group containing 30 numbers of nauplii. The experiment was carried out in triplicate and the setup used is described as follows:

Group 1 – Control
Group 2 - Infected with pathogenic *A. hydrophila* (10^5 CFU/ml) alone
Group 3 - Treated with AgNPs alone (25 µg/ml)
Group 4 – Infected with *A. hydrophila* (10^5 CFU/ml) and treated with AgNPs (25 µg/ml)

The experiment was performed in 24 well plates with 5 ml of seawater (30 ppt salinity) for 48 h to evaluate the biocompatibility and antibacterial efficacy of AgNPs. *A. hydrophila* (10^5 CFU/ml) and AgNPs with concentration of 25 µg/ml were used in this experiment. The survival rate of *Artemia* nauplii was noted over the period of 48 h at 6 h interval and the survival rate was calculated using the below mentioned formula:

$$\text{Survival rate (\%)} = \frac{\text{Number of live nauplii at the 6h intervals}}{\text{Number of nauplii at the time of inoculation}}$$

At the end of experiment, the morphological variation of *Artemia* nauplii in different groups were observed under 10X magnification using inverted phase contrast microscope.

Results and Discussion

Synthesis of silver nanoparticles

The plant, *A. squamosa*, commonly called as "sugar apple or custard apple" is a semi deciduous small tree belonging to the family Annonaceae, comprising of roughly 135 genera and 2300 species. It is mostly cultivated in America, Asia, and Brazil for its edible fruits. It possesses a wide variety of biological and pharmaceutical properties and is widely used in traditional systems of medicine[20,21]. Different parts of *A. squamosa* were used to produce different metallic nanoparticles having biological activities such as anti-cancer, anti-oxidant and insecticidal activity[22-24]. The solution containing silver nitrate and leaf extract gradually changes to dark brown color within 12 h indicating the nanoparticle formation. Reduction and stabilization of silver ions can be accomplished with the biomolecules such as proteins, enzymes, amino acids, alkaloids, saponins, tannins, polysaccharides, phenolics, flavonoids, terpenoids and vitamins[25]. Thus the green synthesis of AgNPs reduce the negative effect of the toxic chemicals on the environment and organisms.

Characterization of silver nanoparticles

UV–Vis spectroscopy is an important and simplest technique to confirm the synthesis of nanoparticles. Maximum absorption peak of colloidal AgNPs were recorded at 425 nm due to surface plasma resonance caused by the free electrons of the silver nanoparticle (Fig. 1a). The results of the present study corroborates with AgNPs synthesized using *A. squamosa* peel extract which recorded the absorbance peak at 420 nm[22]. The XRD data recorded four intense peaks corresponding to 111, 200, 222 and 311 planes of face-centered cubic phase silver (Fig. 1b). The XRD spectrum matches with the standard JCPDS File No. 04-0783. The HR-TEM images show spherical particles in the range of 15-25 nm (Figs. 1c-e). The selected area electron diffraction (SAED) pattern of AgNPs is shown in Figure 1f. Synthesis and properties of AgNPs largely depend on the reducing agent as well as on the physical parameters such as temperature, pH and precursor concentration[22]. AgNPs of different size and shapes were obtained earlier under ambient temperature with different leaf extracts *viz*., *Mangifera indica* (50–65 nm; ovular), *Eucalyptus tereticornis* (60–150 nm; oval), *Carica*

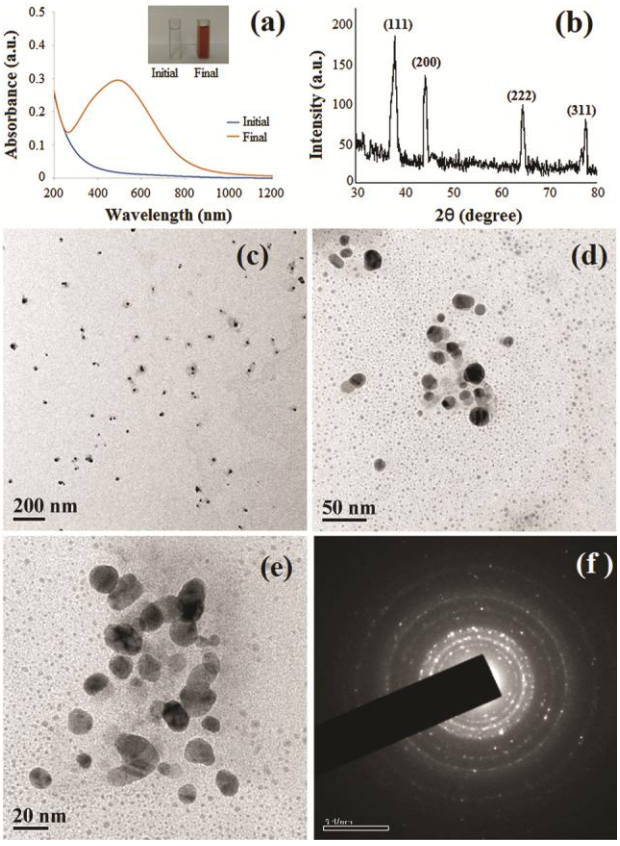

Fig. 1 — (a) UV–Visible spectrum of green synthesized silver nanoparticles using *A. squamosa*. Colour change of the silver nitrate solution indicating the synthesis of silver nanoparticle is shown inside; (b) XRD pattern of AgNPs; (c-e) HRTEM and (f) SAED pattern of green synthesized AgNPs

papaya (25–40 nm) and *Musa paradisiaca* (10–50 nm; round and irregular)[26].

The Energy Dispersive Spectroscopy (EDS) spectrum of AgNPs shows peaks of silver and copper (Fig. 2a). Copper peaks are due to the copper grid used for TEM analysis. Dynamic light scattering (DLS) analysis showed the particles in the range of 5-50 nm (Fig. 2b). Zeta potential determines the surface charge indicating the stability of AgNPs. It was stated that the particles with zeta values greater than ±25 mV are considered stable[27,28]. The zeta potential of AgNPs in the present study was −12.7 mV (Fig. 2c). This suggested that the particles are negatively charged and are moderately stable NPs. The FT-IR spectrum of biosynthesized AgNPs (Fig. 3a) showed strong absorption peak at 3420 cm^{-1} representing the O-H stretching of alcohols and phenols. The peak at 2916 cm^{-1} corresponds to the C-H stretching vibrations of alkenes. The peak at 2848 cm^{-1} and 2360 cm^{-1} are due to O-H stretch of carboxylic acids while the peak at 1615 cm^{-1} is due to C=C of the stretching alkenes. The peak at 1384 cm^{-1}, represents the N-O stretching of nitro compounds. The minor peak at 1042 cm^{-1} is assigned to C-N stretching of aliphatic amines. The alkenes and phenolic compounds in plant extract have been

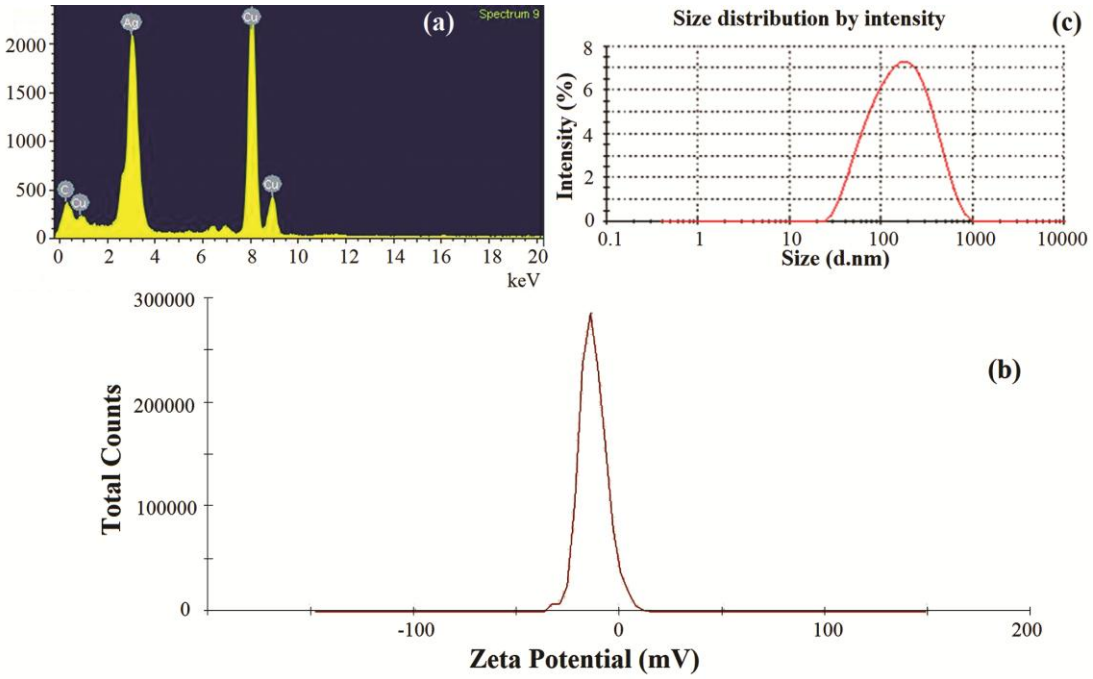

Fig. 2 — (a) EDS spectrum, (b) DLS spectrum, and (c) Zeta Potential analysis of AgNPs

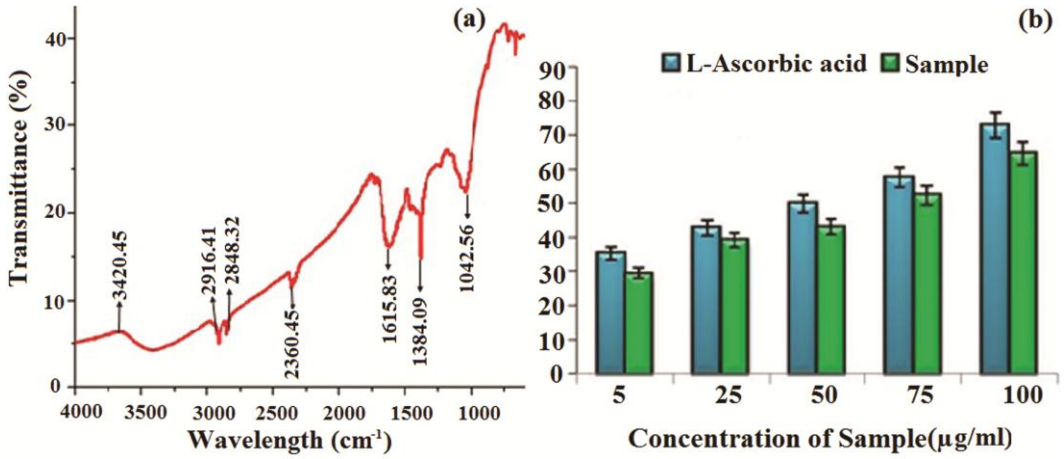

Fig. 3 — (a) FTIR spectrum of AgNPs, and (b) Antioxidant (DPPH) assay of AgNPs

suggested to play a role in the reduction and stabilization of AgNPs. Moreover, phenols (flavonoids) and proteins present in the *A. squamosa* leaf extract are involved in the reduction process. FT-IR revealed that the different functional groups of compounds present in the leaf extract coat the surface of AgNPs[23].

***In vitro* antioxidant activity**

Antioxidants offer defense against oxidative damage and protects organism from stress. Now different methods are available to measure the total antioxidant activity; wherein, DPPH is commonly used to measure the free radical scavenging activity. The DPPH radical scavenging activity of AgNPs was determined in comparison with standard L-ascorbic acid (Fig. 3b). DPPH radical scavenging activity was comparatively lower than that of L-ascorbic acid (standard). However, among the different concentrations tested, the highest scavenging activity (64.62 ± 3.4 %) was obtained at 100 µg/ml and the lowest scavenging effect (29.35 ± 1.5 %) was observed at 5 µg/ml. A dose dependent antioxidant activity of AgNPs was observed.

***In vitro* antibacterial assessment**

Silver nanoparticles have been used in the various fields due to their antimicrobial properties[29]. The synthesized AgNPs showed excellent antibacterial activity against *A. hydrophila* (28 mm zone of inhibition) (Fig. 4). Nanoparticles were reported to interact with building elements of the bacterial membrane causing structural changes, degradation of cell wall and finally leading to cell death[30].

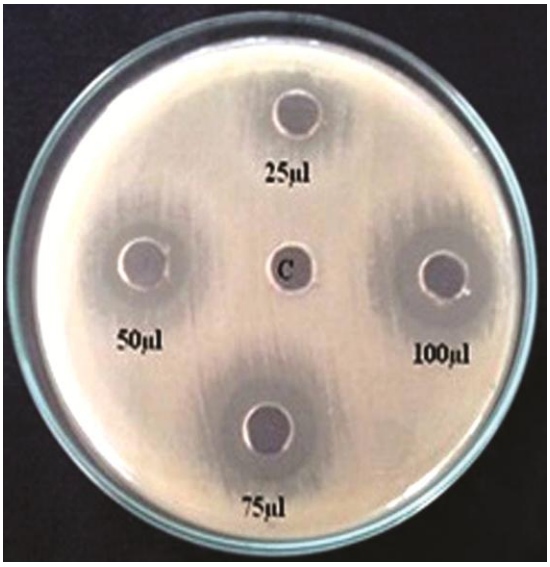

Fig. 4 — Agar well diffusion method showing the antibacterial efficacy of AgNPs against *A. hydrophila*

The AgNPs synthesized using *A. squamosa* leaf extract showed highest inhibition activity against the *A. hydrophila* at 100 µg/ml. The *A. hydrophila* colonies were reduced with increasing concentration of AgNPs (Fig. 5). The antibacterial activity of AgNPs depends on particle size and composition[29]. AgNPs tend to disrupt the outer membrane permeability and shatter the respiratory cycle.

AgNPs has also been reported to exhibit various potent pharmacological activities including antimicrobial activity against *Staphylococcus aureus*, *Salmonella typhi*, *Bacillus subtilis*, *Escherichia coli*[31], and *Mycobacterium smegmatis*[32]; larvicidal activity against larvae of *A. aegypti*[33], and *Culex quinquefasciatus*[34]; anticancer activity against human breast cancer cells (MCF-7)[35], HeLa cancer cell lines[36], A375 skin melanoma cells[37], and in wound healing activity[38].

***In vivo* challenge of *Artemia* nauplii**

Artemia nauplii is recommended as one of the trial organism for acute toxicity testing by US Environmental Protection Agency. The genus *Artemia* has numerous advantages over other model organisms for toxicity assays including easy availability, wide distribution, economic, adaptability to diverse conditions and cysts remain viable for years in dry storage[39]. *Artemia* nauplii assay is rapid (24-48 h), economical, can be maintained easily and simple. Studies based on the toxicity effect of nanomaterials on *A. salina* is limited[40,41]. AgNPs treated group did not show any mortality of *Artemia* nauplii, whereas a higher rate of mortality was observed in control group which was exposed to the pathogen only *(A. hydrophila)*. Hence, the present study clearly

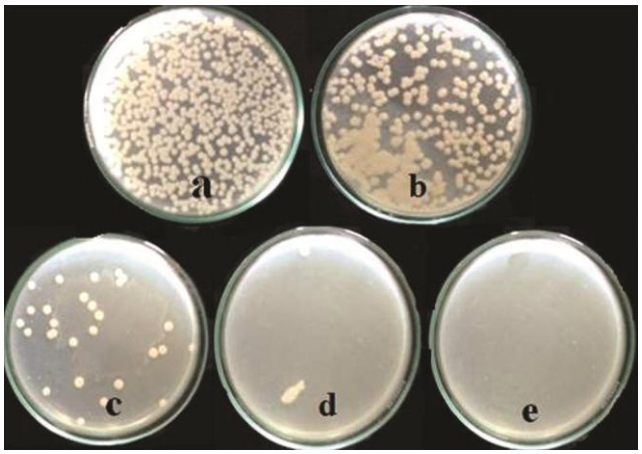

Fig. 5 — Agar bioassay showing the effect of concentration of AgNPs: (a) 0 µg/ml, (b) 25 µg/ml, (c) 50 µg/ml, (d) 75 µg/ml, and (e) 100 µg/ml; on *A. hydrophila* growth

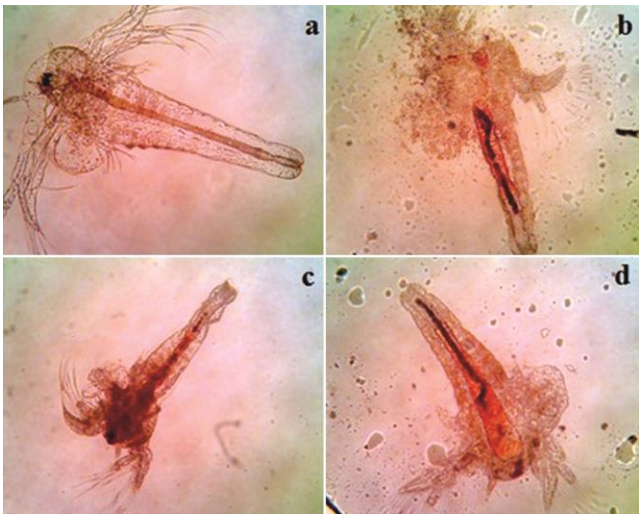

Fig. 6 — *In vivo* study: (a) Group 1 – Control (no treatment); (b) Group 2 – Infected with *A. hydrophila* alone; (c) Group 3 – Treated with 25 µg/ml AgNPs, and (d) Group 4 – infected with *A. hydrophila* and treated with AgNPs

demonstrates the biocompatibility of AgNPs in napulii (I^{st} instar). Silver nanoparticles didn't cause any significant toxicity in *Artemia* nauplii within 48 h compared to *A. hydrophila* (control) (Fig. 6). The microscopic images confirmed the accumulation of AgNPs inside the gut of *Artemia*. In Group II, *Artemia* treated with *A. hydrophila* alone exhibited complete mortality within 24 hrs. Whereas no mortal effect was observed in *Artemia* treated with AgNPs alone in Group III. Interestingly, Group IV infected with *A. hydrophila* and treated with AgNPs showed less mortality than Group-II. Thus, the synthesized AgNPs confirmed the antibacterial effect against *A. hydrophila* (Fig. 6) and also proved to be non-toxic to *Artemia* nauplii.

Conclusion

The present study demonstrates green synthesis of AgNPs using the aqueous *A. squamosa* leaf extract. The synthesized AgNPs are crystalline and are more stable in size range of 15-25 nm. The synthesized AgNPs showed a better antioxidant activity. The results of the *in-vivo* studies confirmed the reduction in the virulence of *A. hydrophila* and enhancement in the survival rate of *Artemia.* The present study proved that the green synthesized AgNPs using *A. squamosa* plant extract are cost effective, non-toxic, eco-friendly and can be applied to tackle the pathogens in aquaculture industry.

Acknowledgements

The authors are thankful to the RUSA Scheme Phase 2.0 grant [F-24-51/2014–U, Policy (TN Multi-Gen), Department of Education, Govt. of India. Dt. 09.10.2018]. Authors also thank the authorities of Alagappa University, Karaikudi for RUSA 2.0 Post-doctoral Fellowship.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

KM: Conceptualization, formal analysis, and writing- original draft preparation; DD and NMV: Formal analysis; EK: Supervision; BM: Investigation; and RK: Writing- reviewing and editing.

References

1 USFDA, *Bad Bug Book - Foodborne Pathogenic Microorganisms and Natural Toxins Handbook*, 2nd edn, (Center for Food Safety and Applied Nutrition, U.S.A.), 2012, pp. 292.

2 van der Marel M, Schroers V, Neuhaus H & Steinhagen D, Chemotaxis towards, adhesion to, and growth in carp gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio* L., *J Fish Dis*, 31 (5) (2008) 321-330. https://doi.org/10.1111/j.1365-2761.2008.00902.x

3 Son R, Rusul G, Sahilah A M, Zainuri A, Raha A R, *et al*., Antibiotic resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured fish, Telapia (*Telapia mossambica*), *Lett Appl Microbiol*, 24 (6) (1997) 479-482. https://doi.org/10.1046/j.1472-765x.1997.00156.x

4 Das B, Samal S, Samantaray B, Sethi S, Pattnaik P, *et al*., Antagonistic activity of cellular components of *Pseudomonas* species against *Aeromonas hydrophila*, *Aquaculture*, 253 (1-4) (2006) 17-24. https://doi.org/10.1016/j.aquaculture.2005.01.028

5 Islam N & Miyazaki K, An empirical analysis of nanotechnology research domains, *Technovation*, 30 (4) (2010) 229-237. https://doi.org/10.1016/j.technovation.2009.10.002

6 Zook J M, Maccuspie R I, Locascio L E, Halter M D & Elliott J T, Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity, *Nanotoxicology*, 5 (4) (2011) 517-530. https://doi.org/10.3109/17435390.2010.536615

7 Nel A, Xia T, Madler L & Li N, Toxic potential of materials at the nanolevel, *Science*, 311 (5761) (2006) 622-627. https://doi.org/10.1126/science.1114397

8 Duan H, Wang D & Li Y, Green chemistry for nanoparticle synthesis, *Chem Soc Rev*, 44 (16) (2015) 5778-5792. https://doi.org/10.1039/c4cs00363b.

9 Hashemi S & Givianrad M H, Biosynthesis and characterization of silver nanoparticles by using brown marine seaweed *Nizimuddinia zanardinii*, *Indian J Geo-Mar Sci*, 47 (12) (2018) 2497-2503.

10 Hamed M R, Givianrad M H & Moradi A M, Green synthesis of silver nanoparticles using marine sponge

Axinella sinoxia, *Indian J Geo-Mar Sci*, 46 (1) (2017) 125-130.

11 Jinu U, Jayalakshmi N, Anbu A S, Mahendran D, Sahi S, *et al*., Biofabrication of cubic phase silver nanoparticles loaded with phytochemicals from *Solanum nigrum* leaf extracts for potential antibacterial, antibiofilm and antioxidant activities against mdr human pathogens, *J Clust Sci*, 28 (1) (2017) 489–505. https://doi.org/10.1007/s10876-016-1125-5

12 Gunasekaran K, Nirmala M, Raja K & Saravanakumar A, Characterization and application of biosynthesized silver nanoparticles from *Melia dubia* leaves, *Indian J Geo-Mar Sci*, 46 (8) (2017) 1715-1720.

13 Tamuly C, Hazarika M, Borah S C, Das M R & Boruah M P, In situ biosynthesis of Ag, Au and bimetallic nanoparticles using *Piper pedicellatum* C.DC: Green chemistry approach, *Colloids Surf B*, 102 (2013) 627-634. https://doi.org/10.1016/j.colsurfb.2012.09.007

14 Zuas O, Hamim N & Sampora Y, Bio-synthesis of silver nanoparticles using water extract of *Myrmecodia pendan* (Sarang Semut plant), *Mater Lett*, 123 (2014) 156-159. https://doi.org/10.1016/j.matlet.2014.03.026.

15 Thirunavoukkarasu M, Balaji U, Behera S, Panda P K & Mishra B K, Biosynthesis of silver nanoparticle from leaf extract of *Desmodium gangeticum* (L.) DC. and its biomedical potential, *Spectrochim Acta A Mol Biomol Spectrosc*, 116 (2013) 424-427. https://doi.org/10.1016/j.saa.2013.07.033

16 Mittal A K, Bhaumik J, Kumar S & Banerjee U C, Biosynthesis of silver nanoparticles: Elucidation of prospective mechanism and therapeutic potential, *J Colloid Interface Sci*, 415 (2014) 39-47. https://doi.org/10.1016/j.jcis.2013.10.018.

17 Kora A J, Sashidhar R B & Arunachalam J, Aqueous extract of gum olibanum (*Boswellia serrata*): A reductant and stabilizer for the biosynthesis of antibacterial silver nanoparticles, *Process Biochem*, 47 (10) (2012) 1516-1520. https://doi.org/10.1016/j.procbio.2012.06.004.

18 Brackman G, Defoirdt T, Miyamoto C, Bossier P, Van Calenbergh S, *et al*., Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR, *BMC Microbiol*, 8 (1) (2008) p. 149. https://doi.org/10.1186/1471-2180-8-149

19 Ravi A V, Musthafa K S, Jegathammbal G, Kathiresan K & Pandian S K, Screening and evaluation of probiotics as a biocontrol agent against pathogenic Vibrios in marine aquaculture, *Lett Appl Microbiol*, 45 (2) (2007) 219-223. https://doi.org/10.1111/j.1472-765X.2007.02180.x

20 Raj D S, Vennila J J, Aiyavu C & Panneerselvam K, The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaves on experimentally induced liver injury in Swiss albino mice, *Int J Integr Biol*, 5 (3) (2009) 182-186.

21 Kaleem M, Medha P, Ahmed Q U, Asif M & Bano B, Beneficial effects of *Annona squamosa* extract in streptozotocin-induced diabetic rats, *Singapore Med J*, 49 (10) (2008) 800-804.

22 Kumar R, Roopan S M, Prabhakarn A, Khanna V G & Chakroborty S, Agricultural waste *Annona squamosa* peel extract: biosynthesis of silver nanoparticles, *Spectrochim Acta A Mol Biomol Spectrosc*, 90 (2012) 173-176. https://doi.org/10.1016/j.saa.2012.01.029

23 Vivek R, Thangam R, Muthuchelian K, Gunasekaran P, Kaveri K, *et al*., Green biosynthesis of silver nanoparticles from *Annona squamosa* leaf extract and its in vitro cytotoxic effect on MCF-7 cells, *Process Biochem*, 47 (12) (2012) 2405-2410. https://doi.org/10.1016/j.procbio.2012.09.025

24 Roopan S M, Bharathi A, Kumar R, Khanna V G & Prabhakarn A, Acaricidal, insecticidal, and larvicidal efficacy of aqueous extract of *Annona squamosa* L peel as biomaterial for the reduction of palladium salts into nanoparticles, *Colloids Surf B*, 92 (2012) 209-212. https://doi.org/10.1016/j.colsurfb.2011.11.044

25 Kulkarni N & Muddapur U, Biosynthesis of Metal Nanoparticles: A Review, *J Nanotechnol*, 2014 (2014) p. 510246. https://doi.org/10.1155/2014/510246.

26 Firdhouse M J & Lalitha P, Biosynthesis of silver nanoparticles and its applications, *J Nanotechnol*, 2015 (2015) p. 829526. https://doi.org/10.1155/2015/829526.

27 Rajendran K, Karunagaran V, Mahanty B & Sen S, Biosynthesis of hematite nanoparticles and its cytotoxic effect on HepG2 cancer cells, *Int J Biol Macromol*, 74 (2015) 376-381. https://doi.org/10.1016/j.ijbiomac.2014.12.028.

28 Rajendran K & Sen S, Optimization of process parameters for the rapid biosynthesis of hematite nanoparticles, *J Photochem Photobiol B*, 159 (2016) 82-87. https://doi.org/10.1016/j.jphotobiol.2016.03.023

29 Feng Q L, Wu J, Chen G Q, Cui F Z, Kim T N, *et al*., A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*, *J Biomed Mater Res*, 52 (4) (2000) 662-668. https://doi.org/10.1002/1097-4636(20001215)52:4<662::aid-jbm10>3.0.co;2-3.

30 Sondi I & Salopek-Sondi B, Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria, *J Colloid Interf Sci*, 275 (1) (2004) 177-1782. https://doi.org/10.1016/j.jcis.2004.02.012

31 Vithiya K, Kumar R & Sen S, Antimicrobial activity of biosynthesized silver oxide nanoparticles, *J Pure Appl Microbiol*, 8 (4) (2014) 3263-3268.

32 Daniel S C G K, Banu B N, Harshiny M, Nehru K, Sankar Ganesh P, *et al*., Ipomea carnea-based silver nanoparticle synthesis for antibacterial activity against selected human pathogens, *J Exp Nanosci*, 9 (2) (2014) 197-209. https://doi.org/10.1080/17458080.2011.654274.

33 Suganya G, Karthi S & Shivakumar M S, Larvicidal potential of silver nanoparticles synthesized from *Leucas aspera* leaf extracts against dengue vector *Aedes aegypti*, *Parasitol Res*, 113 (5) (2014) 1673-1679. https://doi.org/10.1007/s00436-013-3718-3

34 Haldar K M, Haldar B & Chandra G, Fabrication, characterization and mosquito larvicidal bioassay of silver nanoparticles synthesized from aqueous fruit extract of putranjiva, *Drypetes roxburghii* (Wall.), *Parasitol Res*, 112 (4) (2013) 1451-1459. https://doi.org/ 10.1007/s00436-013-3288-4.

35 Sathishkumar G, Gobinath C, Wilson A & Sivaramakrishnan S, Dendrophthoe falcata (L.f) Ettingsh (Neem mistletoe): a potent bioresource to fabricate silver nanoparticles for anticancer effect against human breast cancer cells (MCF-7), *Spectrochim Acta A Mol Biomol Spectrosc*, 128 (2014) 285-290. https://doi.org/10.1016/j.saa.2014.02.096

36 Rajasekharreddy P & Rani P U, Biofabrication of Ag nanoparticles using Sterculia foetida L. seed extract and their toxic potential against mosquito vectors and HeLa cancer

cells, *Mater Sci Eng C*, 39 (2014) 203-212. https://doi.org/10.1016/j.msec.2014.03.003.

37 Das S, Das J, Samadder A, Bhattacharyya S S, Das D, *et al*., Biosynthesized silver nanoparticles by ethanolic extracts of *Phytolacca decandra, Gelsemium sempervirens, Hydrastis canadensis* and *Thuja occidentalis* induce differential cytotoxicity through G2/M arrest in A375 cells, *Colloids Surf B*, 101 (2013) 325-336. https://doi.org/10.1016/j.colsurfb.2012.07.008.

38 Rigo C, Ferroni L, Tocco I, Roman M, Munivrana I, Gardin C, *et al*., Active silver nanoparticles for wound healing, *Int J Mol Sci*, 14 (3) (2013) 4817-4840. https://doi.org/10.3390/ijms14034817

39 Nunes B S, Carvalho F D, Guilhermino L M & Van Stappen G, Use of the genus Artemia in ecotoxicity testing, *Environ Pollut*, 144 (2) (2006) 453-462. https://doi.org/10.1016/j.envpol.2005.12.037.

40 Ates M, Daniels J, Arslan Z & Farah I O, Effects of aqueous suspensions of titanium dioxide nanoparticles on *Artemia salina*: assessment of nanoparticle aggregation, accumulation, and toxicity, *Environ Monit Assess*, 185 (4) (2013) 3339-3348. https://doi.org/10.1007/s10661-012-2794-7.

41 Pretti C, Oliva M, Pietro R D, Monni G, Cevasco G, *et al*., Ecotoxicity of pristine graphene to marine organisms, *Ecotoxicol Environ Saf*, 101 (2014) 138-145. https://doi.org/10.1016/j.ecoenv.2013.11.008.