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ORIGINAL ARTICLE

Phytoextracts-Synthesized Silver Nanoparticles Inhibit Bacterial Fish Pathogen *Aeromonas hydrophila*

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Abstract Fish disease is a major stumbling block towards sustainable growth of the fisheries sector. *Aeromonas hydrophila*, which is a major infectious aquatic pathogen is reportedly the causative agent of ulcers, fin-rot, tail-rot, hemorrhagic septicemia in fish, and has reportedly developed resistance against many of the available antibiotics. In this context, the inhibitory function of silver nanoparticles (AgNPs) against *A. hydrophila* was studied to evaluate its possible application in aquaculture as alternative to antibiotics. AgNPs were synthesized using the leaf extracts of subtropical plants *Mangifera indica* (Mango), *Eucalyptus terticornis* (Eucalyptus), *Carica papaya* (Papaya) and *Musa paradisiaca* (Banana). The absorbance maxima, size range and shape of the AgNPs as characterized by the UV–Vis spectroscopy, high resolution transmission electron microscopy (HR-TEM), and energy dispersive X-ray spectroscopy (EDX) were, *Mangifera*—442, 50–65 nm, ovular; *Eucalyptus*—465, 60–150 nm, oval; *Carica*—442, 25–40 nm, round, irregular; and *Musa*—454, 10–50 nm, round, irregular, respectively. Well-diffusion of these AgNPs for their antimicrobial characteristics exhibited that, the papaya leaf extract synthesized AgNPs had maximum antimicrobial activity at 153.6 μg/ml concentrations, and that from the eucalyptus leaves was least effective. As observed, the potency of the nanoparticles enhanced with the decrease in particle size, from 60–150 nm in eucalyptus to 25–40 nm in papaya. Due to its purely natural sourcing, phytosynthesized AgNPs can be applied as alternative to antibiotics and other biocides as a cost-effective and eco-friendly therapeutic agent against *A. hydrophila* stimulated diseases in aquatic animals.

Keywords *Aeromonas hydrophila* · Silver nanoparticle · Phytosynthesis · Anti-microbial activity

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Introduction

Recent time has seen the highest growth in fisheries among all agricultural sectors, and as an agricultural crop it is widely accepted as a healthy and nutritionally rich food with high protein, quality fat and various micronutrients [1, 2]. As an important agricultural commodity, fish adds value to rural livelihoods, creating employment, generating revenue and above all, ensuring global food security. Fishery creates prolific financial gain compared to any farming activity in aquatic bodies, even only through capture and collection. This sector is also prone to multiple challenges including overexploitation, pollution, climate change and above all, diseases resulting in retarded growth and substantial financial loss.

Among a sizable number of aquatic pathogens, the gram negative *Aeromonas hydrophila* has been recovered from a wide variety of freshwater fishes, and possesses the ability

to grow in both aerobic and anaerobic conditions [3]. It propels outbreak of various fish diseases like ulcers, fin-rot, tail-rot, hemorrhagic septicemia etc. [3, 4]. In human beings, it reportedly causes various water-mediated gastrointestinal infections in children and immunocompromised persons [5]. Its infectivity enhances with environmental pollution, elevation of water temperature and addition of stressors in the aquatic medium.

Successful fish health management practices call for disease prevention rather than their cure. Sterilization techniques do not eliminate all the potential pathogens in the ecological niche of fish. Stressful conditions like suboptimal water quality, poor nutrition and immune suppression provides a conducive environment for opportunistic bacteria like *A. hydrophila* [6], and antimicrobials like antibiotics are generally used in order to prevent disease outbreaks. However, recent report on *A. hydrophila* from various fish tissues revealed that the pathogen had developed resistance to many antibiotics like amoxicillin, ampicillin, lincomycin, novobiocin, oxacillin, penicillin, rifampicin and tetracycline [7], and thus research endeavor to find antibiotics alternatives are gaining momentum [8].

Silver nanoparticles (AgNPs) are medically important and biologically agile nanoparticles. These are being widely used in commercial products for wound dressings, diagnosis, therapeutics, catalysis, biosensing, air and water purification, paints, food packaging [9–14] etc. The Nanomaterial synthesis is the crucial and primary concern in nanotechnology research and application. Many conventional chemical procedures are available to synthesize AgNPs, but numerous supplementary chemicals and capping agents important in synthesis as well as stability enhancement have been found to be environmental pollutants and toxicants, thereby causing discomfiture to the biota. Biosynthesis of AgNPs involving environmentally benign biological substances is a relatively new approach [15–17], which can revolutionize many a sphere of technology. Nanoparticles biosynthesis is a bottom up, easily scalable, eco-friendly and economical technique. Exploiting plants for such synthesis is a novel, economic, simple and fast procedure.

Present study employs leaf extracts of four sub-tropical plants, viz., *Mangifera indica* (Mango), *Musa paradisiaca* (Banana), *Carica papaya* (Papaya) and *Eucalyptus terticornis* (Eucalyptus) for AgNPs synthesis. The selection of the plant species was based on three considerations, such as, their easy aplenty local availability, extract of none of these is a known microbicide, and these belonged to four diverse taxonomic groups. The mango belongs to the order Sapindales, the banana to Zingiberales, the papaya to Brassicales, and the eucalyptus to Myrtales. The two major objectives of the study were, to evaluate the potential of the four leaf-extracts in the synthesis of AgNPs, and to evaluate the antimicrobial potential of the so-synthesized nanoparticles against *A. hydrophila*.

Materials and Methods

Synthesis of AgNPs

Fresh leaves of mango, banana, papaya and eucalyptus were collected locally and washed thoroughly in tap water followed by double distilled water. Leaf broths were prepared by taking 2 g of leaves in 50 ml of deionized water and heating for 45 min at 55 °C. Leaf extracts were collected by decantation and filtration of the broths. 2 ml of each were separately added to 20 ml of 1 mM aqueous solution of silver nitrate, and the reaction mixtures were maintained at ambient temperature till a change of color of the mixture to yellow–brown was observed as a confirmation of nanoparticle formation.

Characterization of AgNPs by UV–Vis Spectra Analysis

The reduction and conversion of Ag^+ ions into AgNPs were evaluated by recording the absorbance of the reaction mixture at 380–520 nm using UV–Vis spectrophotometer (UV-1800, Shimadzu).

Characterizations by HR-TEM and EDX

The sizes of the AgNPs were determined by a HR-TEM (JEM 2100, JEOL) operating at 300 kV and elemental compositions of these were determined by EDX spectroscope (INCAx-Sight, Oxford Instruments) connected to the HR-TEM.

Antimicrobial Assay of the AgNPs

The AgNPs were assayed for antimicrobial potential by well diffusion method. Subculture of *A. hydrophila* was obtained from the Fish Health Management Division of the Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, Bhubaneswar. The bacterium was revived and cultured in LB medium. Five wells of 6 mm dia were made on each dedicated LB agar plate in duplicates. Four wells were loaded with 50 µl of AgNPs, of 153.6, 76.8, 30.7 and 15.3 µg/ml concentrations of original nanoparticles solution, and the fifth well had an equal volume of the pure leaf extract (as control). The cultures were incubated at 37 °C in a thermostatic incubator for 48 h, and the diameter (in mm) of the obvious clear zones was measured indicating a qualitative as well as quantitative performance output.

Results and Discussion

Synthesis of AgNPs

Figure 1 depict that all formulations exhibited color development with different incubation periods. The time interval for color change to brown or yellowish brown signifying AgNPs formation in aqueous solution varied between 3–4 h in eucalyptus and papaya; 4–5 h in mango, and above 6 h in banana. This color changing pattern marking the conversion of bulk silver material into the AgNPs form occurs due to surface plasmon resonance [18]. The time differences observed by these reaction mixtures for color development might be due to the variations in the reducing capabilities of the leaf extracts owing to their varied compositions.

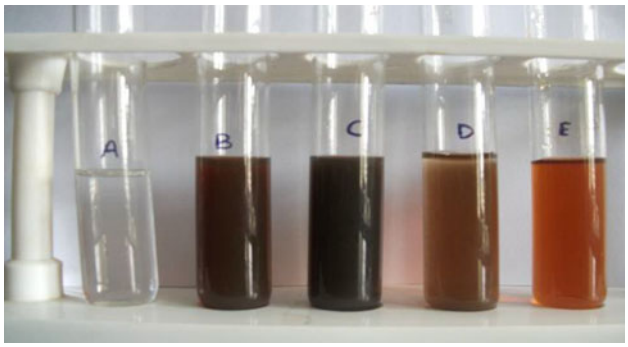

Fig. 1 Photograph showing **a** 1 mM silver nitrate, AgNPs synthesized from **b** Eucalyptus, **c** mango, **d** papaya, **e** banana leaf extracts

Absorbance Analysis

The mango, eucalyptus, banana and papaya leaf extracts synthesized nanoparticles exhibited absorbance maxima at 442, 465, 454 and 442 nm, respectively. Except papaya leaf extract synthesized AgNPs which aggregated after 72–96 h of synthesis, others were relatively stable. All synthesized AgNPs were stable 24 h beyond color change as shown by the graphical representation of absorbance versus wave length with definite time intervals (Fig. 2). Wavelength versus absorption curves showed that with the passage of time, the absorption maxima shifted to 420–450 nm range, attributable to the progressive formation of AgNPs. The broadening of peaks confirms the polydisperse nature of AgNPs.

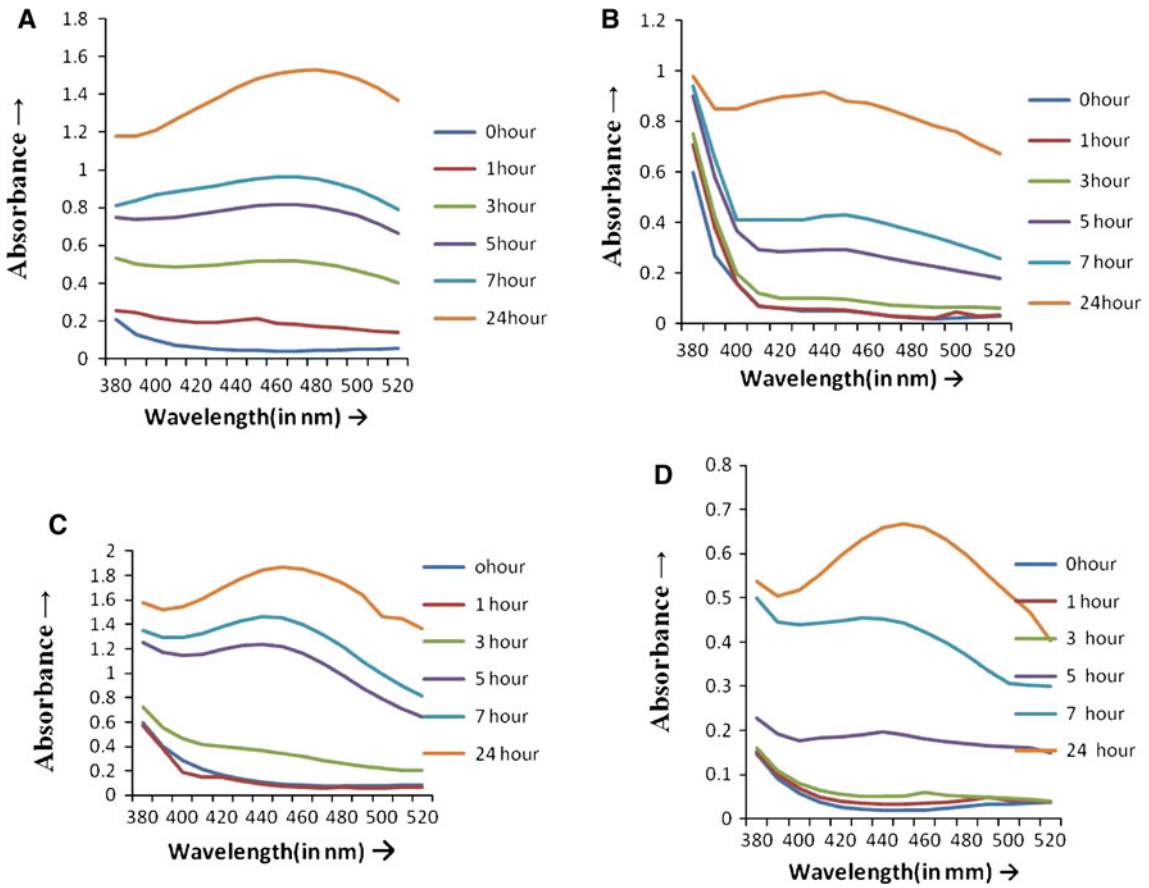

Fig. 2 UV–Vis spectrum of the AgNPs during synthesis using **a** Eucalyptus, **b** mango, **c** papaya and **d** banana leaf extracts

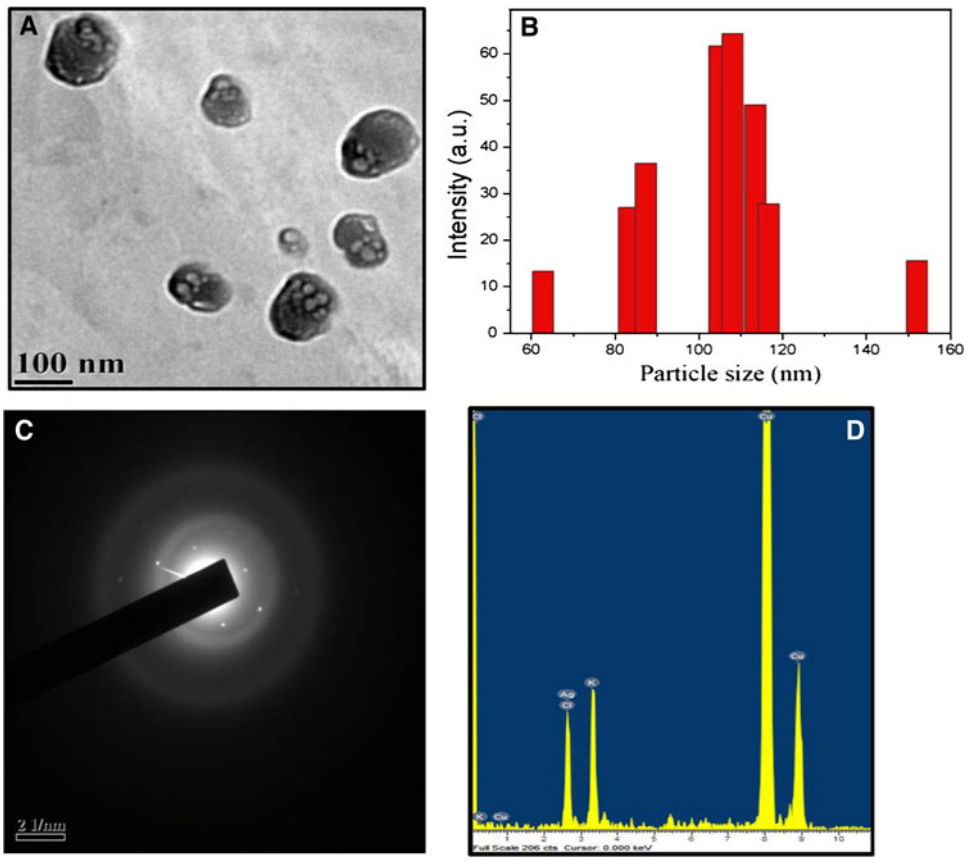

Fig. 3 **a** Size, **b** histogram of the size distribution, **c** HEED and **d** EDX of the AgNPs synthesized from Eucalyptus leaf extract

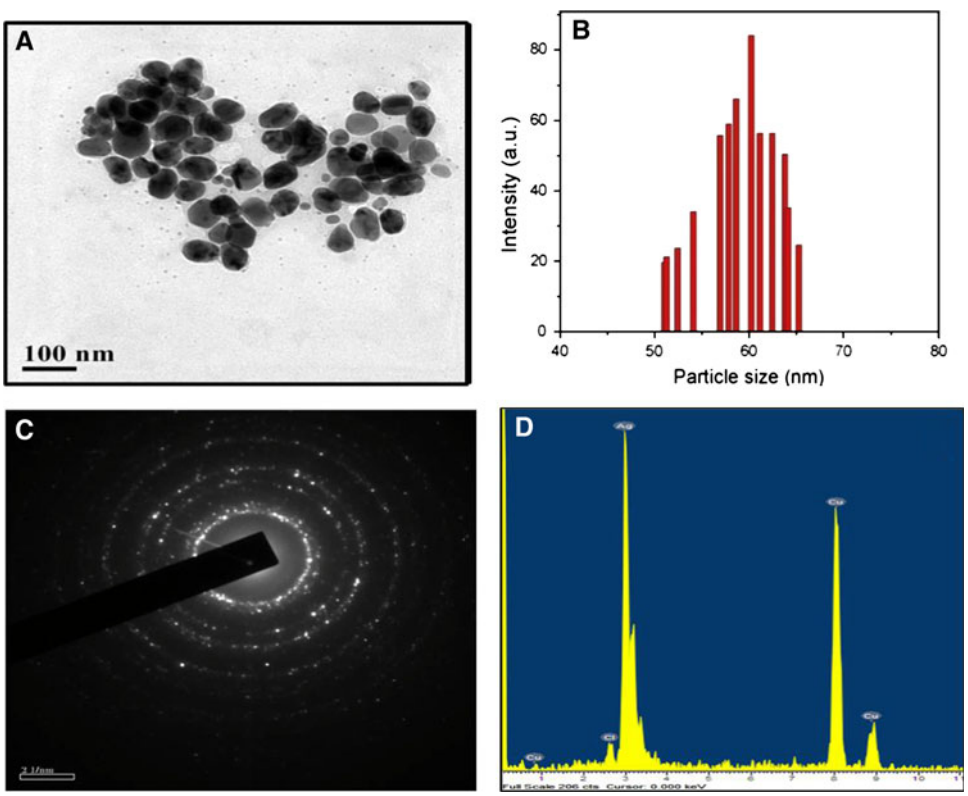

Fig. 4 **a** Size, **b** histogram of the size distribution, **c** HEED and **d** EDX of the AgNPs synthesized from mango leaf extract

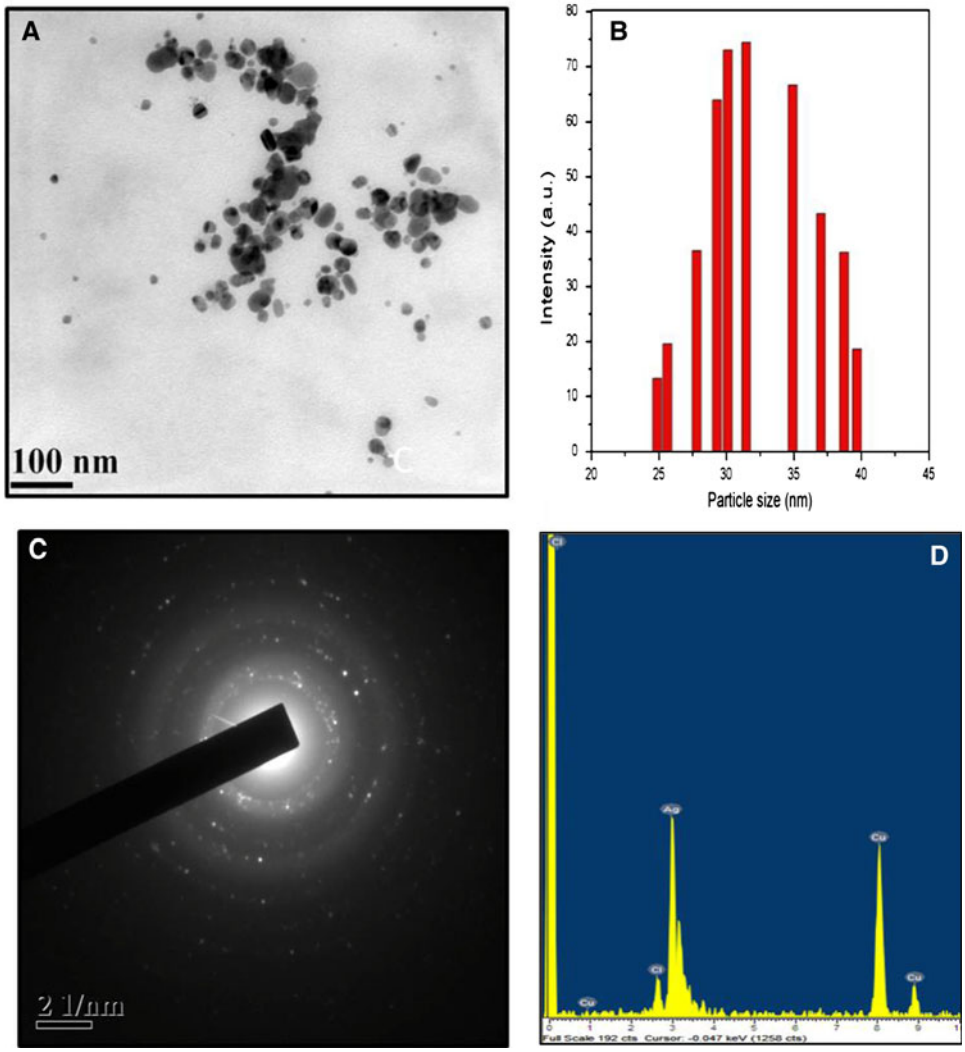

Fig. 5 **a** Size, **b** histogram of the size distribution, **c** HEED and **d** EDX of the AgNPs synthesized from papaya leaf extract

Size and Elemental Composition Analysis

Figures 3, 4, 5 and 6 present the HR-TEM micrograph, SAED, EDX spectra of the AgNPs synthesized from leaf extracts of mango, eucalyptus, papaya and banana, respectively. The HR-TEM micrograph of mango, eucalyptus, papaya and banana leaf extract AgNPs revealed their sizes and shape as 50–65 and ovular, 60–150 and oval, 25–40 and round, and 10–50 nm and irregular, respectively. As the sizes of 'banana' AgNPs were the smallest (10–50 nm) and that of 'eucalyptus' were the largest (60–150 nm), their varying size is attributable to the difference in chemical composition of the phytoextracts. Banana-peel extract synthesized gold nanoparticles (AuNPs) had been reportedly in the range of 300 nm [19]. An encouraging trend in the present study is that, the 'banana' AgNPs synthesized were smaller. An earlier study [20] reported the size and shape of *Eucalyptus hybrida* leaf-extract synthesized AgNPs as 50–150 nm and cubical, while the similar data in the present study were 60–150 nm and oval. Energy dispersive X-ray spectroscopy to determine and confirm the elemental composition of the base element in the AgNPs revealed a sharp peak at 2.9 keV in all the four EDX spectrographs thus confirming the nanoparticles as of silver. Another peak depicts the presence of copper, a basic material in the grid. It was obvious that the phyto-extracts contained different compounds that, besides being reducing agents, act as capping agents. A collective effect of constituent proteins, enzymes, carbohydrates and vitamins might have reduced the silver ions [15–17]. FTIR analyses have brought forth that polyols like hydroxyl flavones and catechins etc. available in the leaves reduce the silver ions during nanoparticle formulation [21].

Antimicrobial Assay

The leaf extract alone (without Ag^{+} treatment) did not have any discernible antimicrobial activity against *A. hydrophila* where as the AgNPs exhibited the same (Fig. 7). The 'papaya' AgNPs showed highest activity and that from

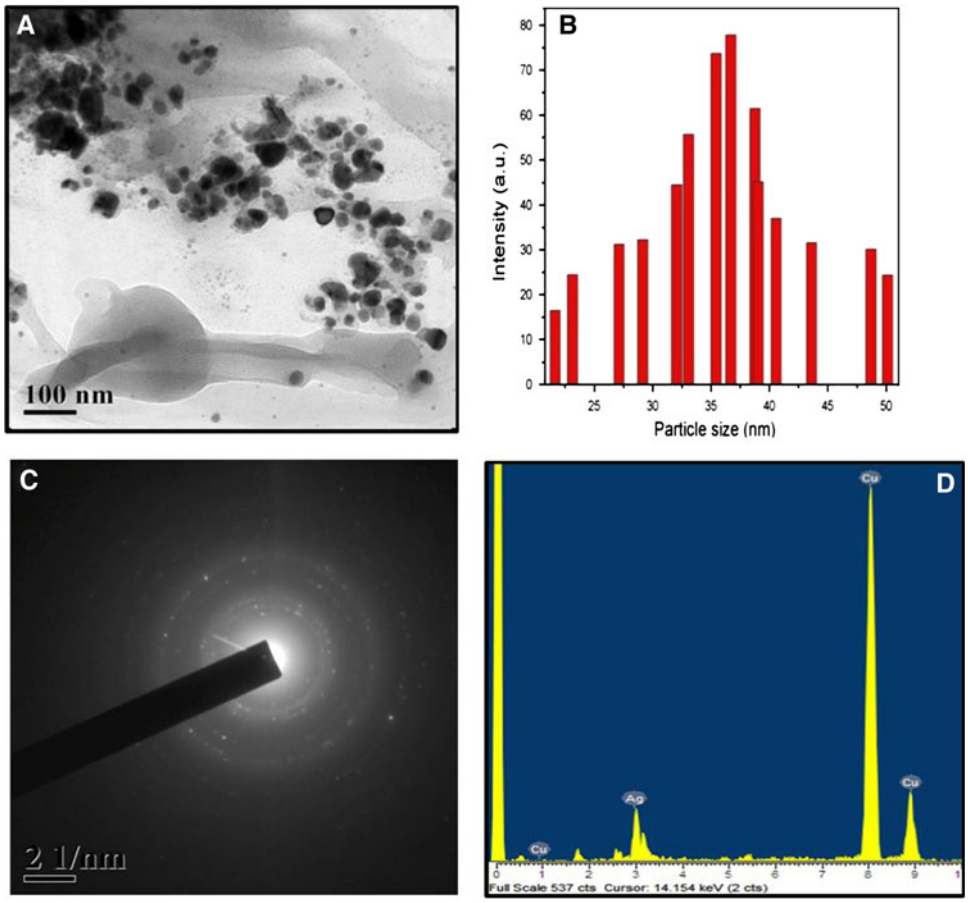

Fig. 6 **a** Size, **b** histogram of the size distribution, **c** HEED and **d** EDX of the AgNPs synthesized from banana leaf extract

eucalyptus exhibited the least. The zones of inhibition of the AgNPs are given in Table 1. From this it was evident that the nanoparticles significantly were active against *A. hydrophila*, possibly due to multiple mechanisms. Ag^{+} reduces phosphate uptake in *Escherichia coli* and drives the efflux of accumulated phosphate as well as of mannitol, succinate, glutamine and proline, and crumple the proton motive force thereby possibly triggering microbial cell death [22]. Studies also suggested that AgNPs could interact with the sulfur-containing proteins and inactivate the amino acids with disulfide bonds. Also, these particles might form pits on the cell wall making it easily permeable and consequently leading to the bacterial kill [23]. Smaller nanoparticles with greater surface area to volume ratio are more deleterious because of wide interactive surface area [24, 25]. Thus, the antimicrobial activity of AgNPs is shape and size dependent. The binding of AgNPs also depends on its available surface area. Truncated and triangular AgNPs are the strongest biocides [26], similar to the findings in the present study (Table 1) at 153.7 μg/ml concentration. Thus, the variation in the antimicrobial activities is attributable to the smaller size of the 'papaya' AgNPs and the larger size of the eucalyptus derived one, in tune with earlier report [27].

1. Regulation of virulence factors is critical for the infection and transmission cycle of a pathogen. The bacterial virulence factor is reportedly under the control of the signaling molecules like acylated homoserine lactone (AHL) produced during a cell density dependent phenomenon of quorum sensing (QS) [28]. *Aeromonas hydrophila* has also been found to producing such molecules for growth in the host [29]. Moreover, it has become increasingly resistant to almost all available recommended antibiotics owing to their evolution of defence mechanisms against antibiotics by exploitation of their large reservoir of genetic variability [27]. Thus, it is important to target these virulence factors with molecular weapons produced by other microbes in order to prevent such bacterial infections and the ensuing diseases [30, 31].

AgNPs reportedly inhibit biofilm formation by disrupting the QS signaling [32]. It also acts intracellular on multiple sites to inactivate critical physiological functions such as cell membrane transport, nucleic acid synthesis and translation, protein folding and function, and electron transport [22, 23]. Hence, as it has to undergo simultaneous mutations in every critical function within a single generation to escape the antimicrobial influence, it is difficult for

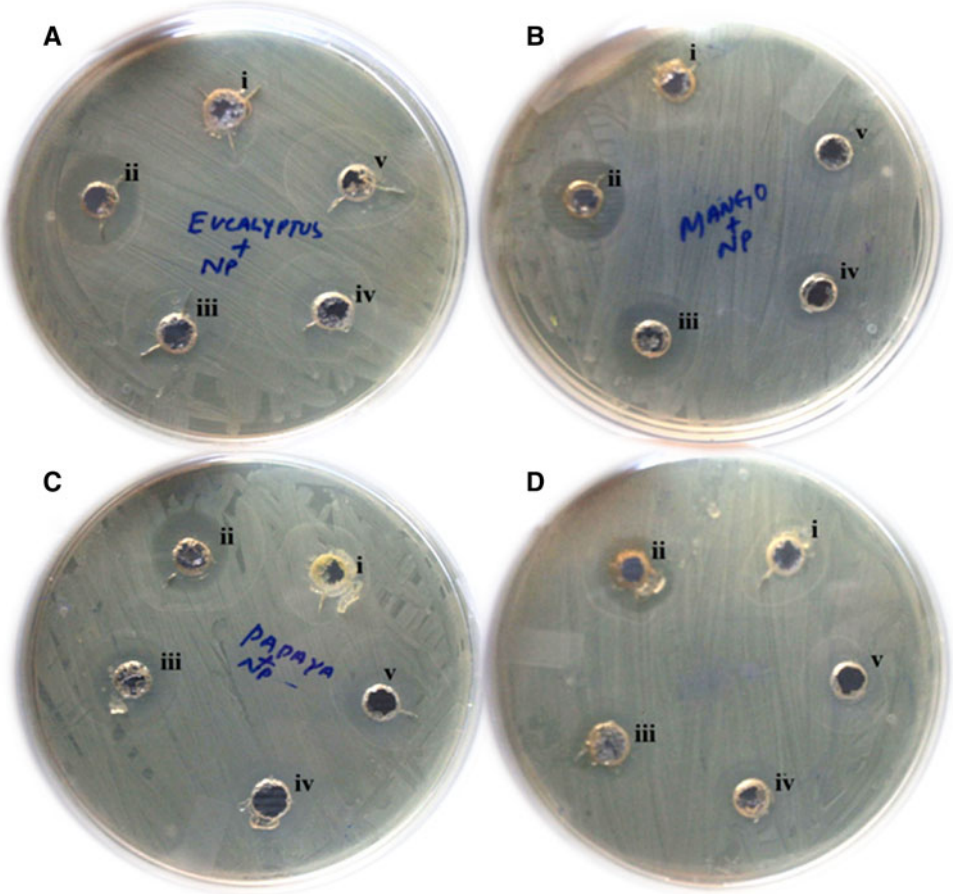

Fig. 7 Antibacterial activity of the AgNPs synthesized using **a** Eucalyptus, **b** mango, **c** papaya and **d** banana leaf extracts, against *Aeromonas hydrophila*. Wells *ii*, *iii*, *iv* and *v* were loaded with 153.6, 76.8, 30.7 and 15.3 μg/ml AgNPs, while well *i* (control) in each case was loaded with the respective pure leaf extract alone

Table 1 Antimicrobial activity of synthesized AgNPs against *Aeromonas hydrophila*

Sample	Diameter (in mm) of the zone of inhibition in various samples[a]			
	Well ii (153.6 μg/ml)	Well iii (76.8 μg/ml)	Well iv (30.7 μg/ml)	Well v (15.3 μg/ml)
Eucalyptus extract + NP	16 ± 0.5	14 ± 0.8	12 ± 0.6	9 ± 0.6
Mango leaf extract + NP	17 ± 0.7	17 ± 0.5	14 ± 0.4	12 ± 0.4
Papaya leaf extract + NP	19 ± 0.6	17 ± 0.8	14 ± 0.6	12 ± 0.5
Banana leaf extract + NP	18 ± 0.7	18 ± 0.4	15 ± 0.5	14 ± 0.4

[a] Well i with control (4 % leaf extract) did not show any zone of inhibition

any microbe to develop resistance against AgNPs [33]. Several phytochemicals are known as quorum quenchers (QQ) that reportedly inhibit QS signaling [34]. So, phyto-synthesized AgNPs can have a cumulative effect of natural and synthetic QQ to be applied in aquaculture as a potential drug candidate and/or disinfectant to treat *A. hydrophila* infections and related diseases.

Research on the biodegradable polymers for controlled release of nanoparticles in field conditions is gaining momentum. Integrative processes for the production of bioenergy and biopolymers are also gaining importance. Novel works on PHB production by a non-photosynthate *Bacillus* under dissimilar culture conditions [35], and inducing nanoparticles production by *B. thuringiensis* in modified physiological conditions [14] have been recently reported. Another recent study comparing the antimicrobial treatments of the packaging films through gamma irradiation and AgNPs impregnation [36] reports that the total aerobic mesophilic bacteria, Enterobacteriaceae, *E. coli* and *Clostridium perfringens* demonstrated greater inhibition zones when irradiated at 4 kGy without AgNPs and at 2 kGy with AgNPs impregnation which further strengthens the antimicrobial opinion about AgNPs.

Conclusion

The phyto-synthesized AgNPs with biocidal activity against fish pathogens can become an asset for fishery and aquaculture industry as a potential alternative to antibiotics [37]. The use of such eco-friendly biosynthesized AgNPs as alternative to chemically synthesized ones would help control chemical toxicity in the environment. Such organically synthesized AgNPs can also be embedded in cemented cisterns, plastic pools or any other erection used in fisheries to provide a sustained infection-free culture environment.

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