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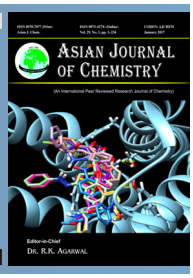

Synthesis and Distribution of Bioinspired Silver Nanoparticles Using Spirulina Extract for Control of *Vibrio parahaemolyticus* Infection in Aquaculture

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In the present study the antibacterial efficacy of green synthesized silver nanoparticles using *Spirulina* extract is reported. Synthesis of silver nanoparticles (AgNPs) was achieved by mixing 15 mL of *Spirulina* extract with 85 mL of 1 mM of silver nitrate solution in a conical flask and incubated at room temperature under stirred condition for 4 days. The synthesized AgNPs were characterized by UV-visible spectroscopy, X-ray diffraction, fourier transform infrared spectroscopy, selected area electron diffraction (SAED), energy dispersive X-ray spectroscopy, field emission scanning electron microscopy and transmission electron microscopy. The biosynthesized silver nanoparticles exhibited a higher antibacterial activity (28 mm) against *Vibrio parahaemolyticus* at 150 µg/mL. Further, synthesized AgNPs reduces the mortality rate of *Artemia nauplii* up to 26.6 % by controlling the *V. parahaemolyticus* infection. This finding confirms the antibacterial potential of *Spirulina* mediated silver nanoparticles against the aquaculture pathogen *V. parahaemolyticus*.

Keywords: *Vibrio parahaemolyticus, Spirulina*, Silver nanoparticles, Antibacterial activity.

INTRODUCTION

Vibriosis is a foremost disease in shrimp and marine finfish aquaculture. This bacterial group causes hazardous health problems in many marine cultivable species including ornamental fishes. *Vibrio* species have been associated with mortalities in iberian tooth carps, milk fish [1] abalone, [2] shrimp [3,4] crab and lobster [5]. The primary species causing vibriosis in marine finfishes and crustaceans are *Vibrio harveyi, Vibrio alginolyticus, Vibrio vulnificus*, *Vibrio anguillarum* and *Vibrio parahaemolyticus*. These *Vibrio* infections cause high mortality and stunted growth in the culture organisms [6]. Generally *V. parahaemolyticus* colonize on the surface of the cultured organism and cause mass mortality, which resulted huge production loss to the farmer. Further it is also responsible for the most common seafood borne gastroenteritis in humans [7]. Application of antibiotics and chemotherapeutic agents are important bacterial disease control measure in the aquaculture industry. The conventional antibiotics have only had partial success in the treatment of aquaculture diseases [8,9] owing to an increase in multidrug resistant, boost the health care cost and hazardous to the environment [10-12]. To keep a sustainable growth pattern in aquatic health management practices one must go ahead of antibiotics. Therefore, there has been increasing pressure for scientists to find out a novel and eco-friendly antibacterial agent to combat against the pathogenic bacteria [13]. Interdisciplinary research has been widened the perspective of material research, drawing new stimulation from biological systems. In recent decades, biologically synthesized silver nanoparticles have been proved to be much superior to the chemical and physical methods also cost effective as well as eco-friendly [14-17]. Earlier reports authenticated that the prokaryotes are the prospective nanoparticle synthesizers [18,19]. However, the use of eukaryotes, especially fungi and algae are potentially exciting, since they secrete large amounts of proteins. Thus, it has many advantages, which increase the productivity, easy to handle and suitable option for the metallic nanoparticle production [20,21].

In recent days, *Spirulina* has been receiving greater attention due to its health benefits. Based on the earlier reports, *Spirulina* has a wide range of pharmacological activities such as antibacterial, antiviral, anticancer, antidiabetics and antioxidant [22-28]. However, limited studies have been focused on nanoparticle applications in the field of aquaculture. Moreover few reports are available on the subject of the AgNPs synthesized using *Spirulina platensis* culture [29-31]. Further, the application of biosynthesized silver nanoparticles to control vibriosis in marine finfishes and crustaceans is still at juvenile stage. Besides the antibacterial activity of green synthesized silver nanoparticles using *Spirulina* extract against *Vibrio* species is unexplored. This

study may have the prospective to solve many unsolved questions related to aquatic animal health and production.

Artemia nauplii is a non selective filter feeding small crustacean [32] newly hatched *Artemia nauplius* has been used as live food source for the marine finfish and shrimp larvae in hatcheries. The short life cycle, high offspring production and easy to culture in the laboratory are the characteristic features of this organism [33]. Furthermore, *Artemia* is also included as one of the trial organism for acute toxicity testing by US Environmental Protection Agency [33-35]. With this competence, *Artemia* appears to be a suitable model species to investigate the antibacterial efficacy and toxicity studies. Therefore, in this study *Artemia* was selected for the *in vivo* analysis. To our best of knowledge few reports are available on biosynthesized silver nanoparticles and its application against aquaculture bacterial pathogens. Besides the effective concentration of biosynthesized AgNPs to control the bacterial pathogens using various type of aquaculture organism is lacking. This study deals with *Spirulina* mediated synthesis of silver nanoparticles and their antibacterial efficacy against the aquaculture pathogen *V. parahaemolyticus* under *in vitro* and *in vivo* conditions.

EXPERIMENTAL

The silver nitrate ($AgNO_3$) and Microbial culture media were procured from HiMedia Laboratories, India. All chemicals and reagents used in this experiment were analytical grade. Sterile double distilled water was used throughout the study. In this study Gram-negative bacterial strain *V. parahaemolyticus* (ATCC-17802) was used as a test organism. *Spirulina* powder was purchased from the Antenna Nutritech Foundation, Madurai, Tamilnadu and commercially available *Artemia* cyst (San Francisco Bay Brand, San Francisco, CA, USA) was used in this study.

Preparation of *Spirulina* aqueous extract: 0.5 g of *Spirulina* powder was weighed and transferred into 250 mL beaker containing 100 mL of distilled water, mixed well and kept in orbital shaker for 6 h at 120 rpm. The aqueous extract was filtered through Whatman No. 1 filter paper. Then the filtered extract was centrifuged at 8000 rpm for 15 min and the supernatant was used to synthesize the silver nanoparticles.

Synthesis of silver nanoparticles: 15 mL of *Spirulina* extract was taken into 250 mL of Erlenmeyer flask. Then the extract was mixed with 85 mL of 1 mM of silver nitrate solution afterward the solution was incubated at room temperature under stirred condition for 4 days. The colour changes occurred in the solution was visually noted. The appearance of yellowish brown colour indicates the formation of AgNPs. The reduced solution was centrifuged at 8000 rpm for 30 min. The supernatant was discarded and the obtained pellets were redispersed in deionized water. The centrifugation process was repeated for three times to remove of any absorbed substances on the surface of silver nanoparticles.

Characterization of nanoparticles: The reduction of pure Ag^+ ions into Ag^0 was monitored by measuring the UV-visible spectra of the solution. UV-visible spectra of the solutions were recorded on UV-visible Spectrophotometer (UV-1800 Shimadzu, Japan) operated at a resolution of 1 nm, between 200 and 800 nm. The Milli-Q water was used as a blank. In order to determine the crystalline nature of particles, lyophilized silver nanoparticles was subjected to X-ray diffraction (XRD) analysis (X'PERT- PRO.PAN analytical Netherland) operating in transmission mode at 40 kV and 30 mA with CuK_α radiation in the scanning range of θ-2θ configuration was selected from 10° to 80°. Further, the data obtained was compared with the Joint Committee on Powder Diffraction Standards (JCPDS) library to account for the crystalline structure of the particle. In order to observe the capping behaviour of biomolecules and possible functional groups present in the *Spirulina* extract for the formation of AgNPs, Fourier transform infrared spectroscopy (FTIR) was performed. Two milligrams of *Spirulina* sample were mixed with 200 mg of KBr (FTIR grade) and pressed into a pellet then placed in the sample holder of FTIR spectra. To obtain good signal to noise ratio, 256 scans of AgNPs were measured within the range of 4000-500 cm^{-1} and the resolution was kept at 4 cm^{-1} in the transmission mode using a Thermo Scientific Nicolet 380 FTIR spectroscopy. To facilitate the morphology and elemental composition of AgNPs, field emission scanning electron microscopy (FESEM) was carried out on a FEG Quanta 250, Czech Republic and to realize the composition energy dispersive X-ray spectroscopy (EDX) (Hitachi S3000H) was performed. In order to analysis the particle size of synthesized nanaparticles, transmission electron microscopy (TEM) analysis was executed on Technite 10 Philips instrument on carbon coated copper grids with an accelerating voltage of 80 Kv.

Screening of antibacterial activity against *Vibrio parahaemolyticus*: The synthesized silver nanoparticles were tested for their antibacterial activity against targeted pathogen *V. parahaemolyticus* by agar well diffusion assay [36]. In brief, the *V. parahaemolyticus* strain was grown overnight at 37°C in a medium containing alkaline peptone water prepared in 10 g L^{-1} (pH 7.8 ± 0.2). The targeted bacterial strain at the density of 10^5 CFU/mL was seeded on the plates containing LB (Luria Bertani) medium. This bacterium was swabbed uniformly on the plates using sterile cotton swabs. In this medium 6 mm diameter wells were made using gel puncture and filled with different concentration of nanoparticles (25, 50, 75, 100, 125 and 150 µg/mL). Positive and negative control also maintained to compare the results. The plates were incubated at 37 °C for 24 h and observed for the zone of inhibition.

In vivo analysis

Culturing *Artemia*: 2 g of Artemia cyst (San Francisco Bay Brand, San Francisco, CA, USA) were decapsulated (removal or softening of the outer membrane by using sodium hypochlorite, without affecting the viability of the embryo) and incubated in 1 L of seawater (30 ppt) in a controlled environment (30 ± 1 °C; 1,500 lux light intensity with aeration) by using a conical plastic container. Freshly hatched *Artemia nauplii* were collected and used for *in vivo* studies [37].

***In vivo* challenging experiment:** The antibacterial potential of silver nanoparticles against the *Artemia nauplii* infected with *V. parahaemolyticus* was determined according to reported method with minor modifications [38]. Briefly, < 24 h old healthy *Artemia nauplii* (30 nos) were introduced into a plastic container contained 25 mL of filtered seawater with the salinity of 30 ppt at room temperature. The *Artemia nauplii* were divided into four groups in triplicates. The concentration of the AgNPs was

selected based on the previous report [39]. The experimental setup as follows:

Group-1 = Control (without any applications); Group-2 = *Artemia nauplii* treated with *V. parahaemolyticus* (10^5 CFU/mL) alone; Group-3 = *Artemia nauplii* treated with AgNPs (5 µg/mL) alone; Group-4 = *Artemia nauplii* treated with *V. parahaemolyticus* (10^5 CFU/mL) and AgNPs (5 µg/mL).

Mortality in each group was recorded after 24 h and the percentage of mortality was calculated by the following formula:

$$\text{Mortality (\%)} = \frac{\text{Number of dead } \textit{Artemia nauplii}}{\text{Initial number of live } \textit{Artemia nauplii}} \times 100$$

Statistical analysis: All results are expressed as mean ± SD. Independent sample student's t test was used to assess the data. A P-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Green synthesis of silver nanoparticles: When the *Spirulina* extract was added to the silver nitrate solution, the reaction was started and colour of the reaction mixture was changed from pale blue to yellowish brown after 72 h, which indicates the formation of silver nanoparticles (Fig. 1). The UV-visible spectrum of silver nanoparticles is shown in Fig. 1. The surface plasmon resonance of the silver nanoparticles reduced by the *Spirulina* aqueous extract was obtained at 431 nm.

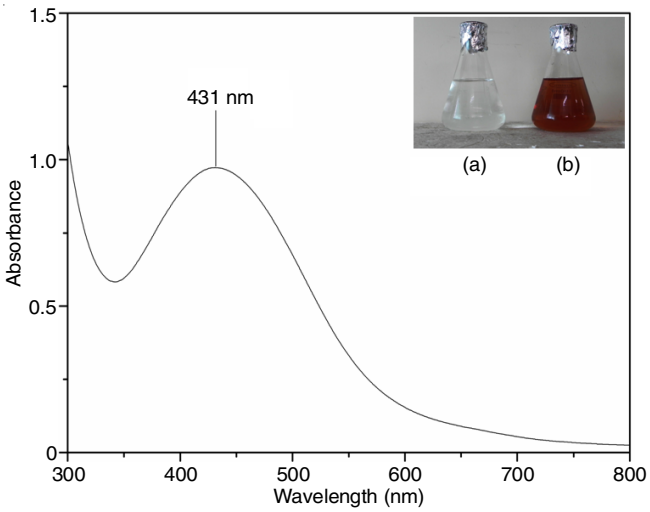

Fig. 1. UV- Visible spectrum of silver nanoparticles synthesized by treating *Spirulina* extract. Inset figure shows the colour of silver nitrate solution (a) before and (b) after adding the *Spirulina* extract

Characterization of silver nanoparticles: Fig. 2 demonstrates the X-ray diffraction pattern of synthesized silver nanoparticles. Four different and significant characteristic peaks were observed at 38.2, 44.3, 64.5 and 77.3 correspond to the lattice planes (111), (200), (220) and (311) which were indexed for face-centered cubic structure of silver. The obtained Bragg peaks are compared with pure crystalline silver published by Joint Committee on Powder Diffraction Standards (File No. 89-3722). Further, sizes of silver crystallite was calculated from broadening planes 111, 200, 220 and 311using Debye Scherrer equation:

$$D = \frac{K\lambda}{\beta \cos\theta}$$

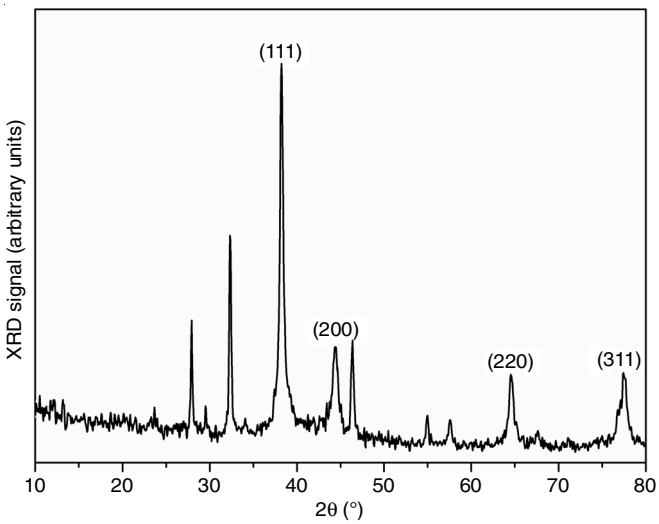

Fig. 2. X-ray diffraction pattern of silver nanoparticles synthesized using *Spirulina* extract

where D is the average particle size, k is the shape factor (constant 0.9), λ is the X-ray wavelength (1.5406 Å), β is the full width at half maximum of the peak (FWHM) and θ is the diffraction angle. The sizes of silver nanoparticles synthesized by *Spirulina* aqueous extract were 20.1, 12.3, 16.4 and 11.3 nm, respectively.

The major biomolecules responsible for the reduction of silver ions (Ag^+) into silver nanoparticles (Ag^0) present in the *Spirulina* extract were identified by FTIR spectroscopy. The IR spectrum of *Spirulina* extract alone exhibited prominent transmission peaks in the range of 3424, 2853, 2924, 1652, 1545, 1451, 1404 and 1082 cm^{-1} (Fig. 3a). The peak at 3424 cm^{-1} was referred as O–H stretching vibration due to presence of alcohols, phenols groups. The corresponding peaks at 2853 and 2924 cm^{-1} due to the C–H stretching vibration presence of alkenes. The peak at 1652 cm^{-1} was responsible for the C=O stretch vibrational mode. The peaks at 1545 cm^{-1} and 1451 cm^{-1} were responsible for N–O asymmetric stretching vibration presence of nitro compounds and C–H bend stretching vibration due the presence of alkenes, respectively. The vibrational mode at 1404 and 1082 cm^{-1} were C-C stretch, aromatics and C–N stretching vibration presence of aliphatic amines present in the *Spirulina* extract. The FTIR spectrum of silver nanoparticles exhibited the distinct peak in the range of 3405 cm^{-1}, 1609 cm^{-1}, 1384 cm^{-1}, 1046 and 824 cm^{-1} which indicates O–H stretching vibrational group, N-H bend amines, N–O stretching vibration mode, C–N stretching vibration mode presence of aliphatic amines and N–H stretching vibration presence of primary, secondary amines, respectively (Fig. 3b). The comparison of both FTIR spectrum, the IR peaks at 2853, 2924 cm^{-1}, (C–H stretching vibration mode) 1652 cm^{-1} (C=O stretch), 1451 cm^{-1}, (C–H bend stretching vibration mode) and 1404 cm^{-1} (C-C stretch, aromatics), were suppressed after the synthesis of silver nanoparticles so these groups are responsible for the reduce the Ag ions to Ag^0 nanopartilces. In addition the peak, 3424 cm^{-1} (O–H stretching vibration) was shifted to lower wavelengths 3405 cm^{-1} this incidence was due to the synthesis of silver nanoparticles.

Fig. 4(a) and (b) illustrates the FESEM and TEM images, which provide a clear idea on the shape of nanoparticles. The

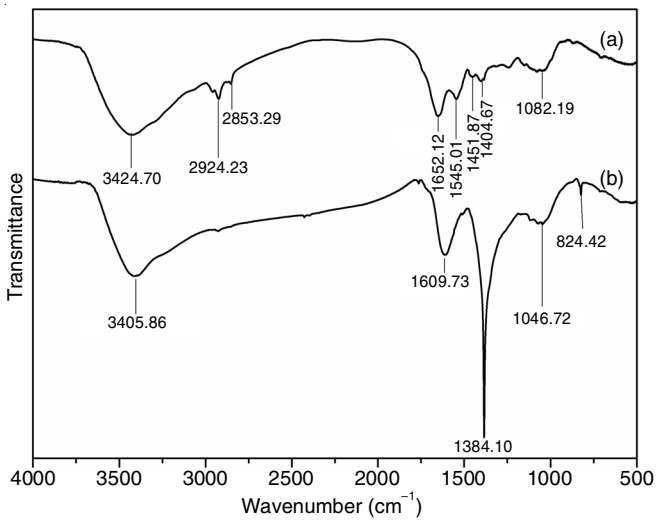

Fig. 3. Fourier transform infrared spectra (a) *Spirulina* extract (b) silver nanoparticles

results showed that the majority of the particles were spherical and oval in shapes with size ranging from 2 to 18 nm. These particles were well distributed without any aggregation. The presence of silver elements in the reaction mixture was confirmed by EDAX spectroscopy analysis. Fig. 4(c) clearly demonstrate presence of strong silver signal with some other peaks, it's may be attributed to proteins and other biomolecules associated with the biomass extract. Selected area electron diffraction (SAED) results proved that the diffraction patterns were directed toward (111), (200), (220) and (311) planes which correspond to the face-centered cubic (FCC) structure of elemental silver. Therefore, it confirms the polycrystalline nature of silver nanoparticles (Fig. 4d).

Agar well diffusion assay: The antibacterial activity of synthesized silver nanoparticles was tested with different concentrations (25, 50, 75, 100, 125 and 150 µg/mL) against *V. parahaemolyticus* is shown in Fig. 5. The AgNPs make strong zone of inhibition, while increasing the concentration

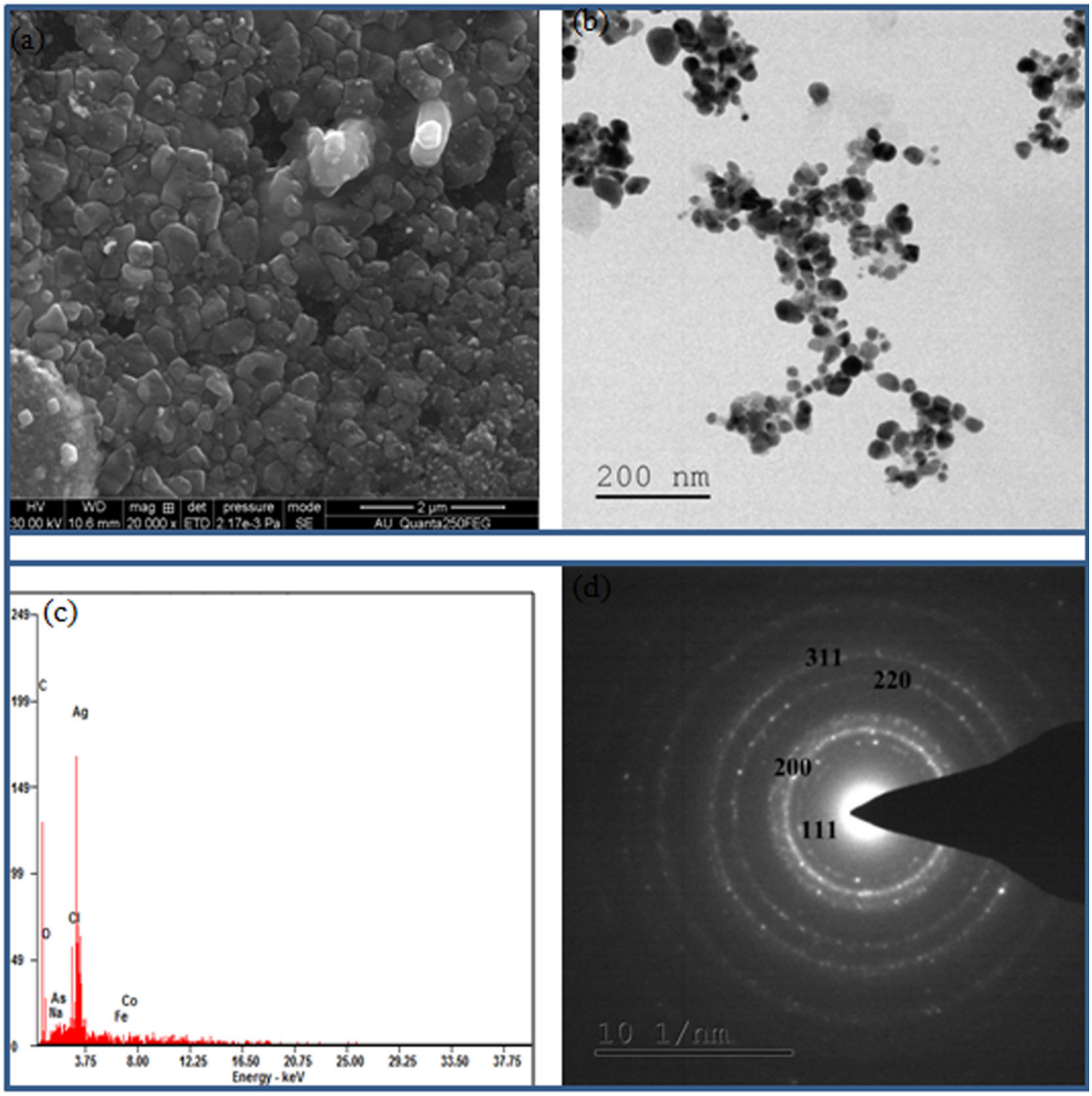

Fig. 4. (a) Field emission scanning electron microscopy image (b) Transmission electron microscopy image (c) Energy dispersive X-ray spectroscopy image showed higher percentage of silver signal. (d) Selected area electron diffraction pattern of *Spirulina* mediated synthesized silver nanoparticles

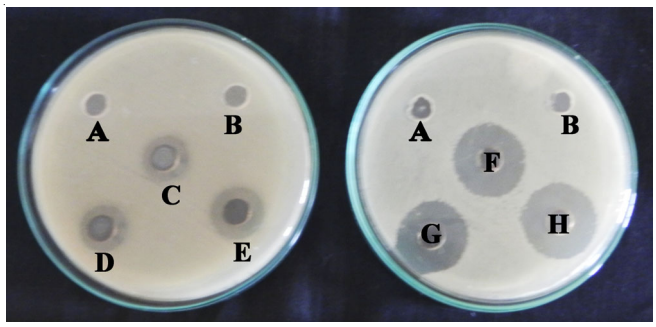

Fig. 5. Dose dependent antibacterial efficacy of *Spirulina* mediated silver nanoparticles against *Vibrio parahaemolyticus* (A) 1 mM silver nitrate solution (B) *Spirulina* aqueous extract (C) Silver nanoparticle 25 µg/mL concentration (D) silver nanoparticle 50 µg/mL concentration (E) silver nanoparticle 75 µg/mL concentration (F) silver nanoparticle 100 µg/mL concentration (G) silver nanoparticle 125 µg/mL concentration (H) silver nanoparticle 150 µg/mL concentration

of nanoparticles the zone of bacterial inhibition also amplified. The highest zone of inhibition (28 mm at 150 µg/mL) and the minimum inhibition 15 mm at 25 µg/mL were observed.

***In vivo* evaluation:** The antibacterial potential of silver nanoparticles against the *Artemia nauplii* infected with *V. parahaemolyticus* is shown Table-1. In this study, complete mortality was observed in *Artemia nauplii* exposed to *V. parahaemolyticus* alone (Group II). However, *Artemia nauplii* treated with *V. parahaemolyticus* (10^5 CFU/mL) and AgNPs at the concentration of 5µg/mL showed 26.6 % mortality (Group IV). AgNPs significantly decreased ($P < 0.005$) the mortality rate in Group IV when compared to Group II, *Artemia nauplii* infected with *V. parahaemolyticus* alone. In Group III, *Artemia nauplii* treated with AgNPs alone showed only 6.6 % of mortality. The above finding clearly shows that the green synthesized silver nanoparticles using *Spirulina* extract exhibited outstanding antibacterial efficacy against *V. parahaemolyticus*.

TABLE-1
ANTI-INFECTIVE POTENTIAL OF SILVER NANOPARTICLES AGAINST *Vibrio parahaemolyticus* TREATED *Artemia nauplii*

Groups	Initial number of *Artemia nauplii*	Number of *Artemia nauplii* dead after 24 h (mean ± SD)	Percentage of mortality after 24 h
Group-1	30	1 ± 0.0	3.3
Group-2	30	30 ± 0.0	100
Group-3	30	2 ± 1.0	6.6
Group-4	30	8 ± 1.5	26.6

Group-1 = *Artemia* alone (negative control); Group-2 = *Artemia nauplii treated with Vibrio parahaemolyticus* (10^5 CFU/mL); Group-3 = *Artemia nauplii* treated with silver nanoparticle (5 µg/mL) serves as positive control; Group-4 = *Artemia nauplii* treated with *Vibrio parahaemolyticus* (10^5 CFU/mL) and AgNPs (5 µg/mL). Each value represents mean ± SD. The mortality percentage of *Artemia nauplii* was significantly different from the control group. The significant difference ($P < 0.005$) in mortality percentage was observed between Group-2 and Group-4.

Synthesis of inorganic materials by means of biological resources like plants, bacteria, fungi and algae as nano scale level have created greater attention among material scientists. The green nanotechnology has many applications in the field of medicine, pharmacy and animal health management. *Spirulina* is widely used in pharmaceuticals and food industries due its medicinal and nutritional value. Moreover *Spirulina* has been used as a biologically active feed additive for humans and animals because of its dietary principles such as 60 to 70 % of protein, rich in amino acid, essential fatty acids, β-carotene, iron and vitamins. Hence, in the present study *Spirulina* has been selected for the synthesis of silver nanoparticles. It is well known fact that silver and its compounds have a broad spectrum of antimicrobial activity [40,41] with lower toxicity to mammalian cells [42]. Next to the viral infection, vibriosis has become an integral part of intensive aquaculture production, which leads to huge production losses in this industry. Furthermore, the effect of *Spirulina* mediated silver nanoparticles against *V. parahaemolyticus* using *Artemia nauplii* is also not been reported. Therefore, the present study was proposed to synthesize the silver nanoparticles using *Spirulina* extract and to evaluate the antibacterial efficacy against the aquaculture pathogen *V. parahaemolyticus* under *in vitro* and *in vivo* conditions.

It was confirmed with various reports that the colour change and appearance of peak at 431 nm corresponds to the absorption intensity steadily increased as a function of time reaction without any shift in the peak position. This peak is due to the excitation of surface plasmon resonance distinctive of AgNPs [43]. Further, this result was comparable with the earlier findings [44] that the *Spirulina platensis* mediated silver nanoparticles exhibit absorption peak at 430. Subsequently, synthesized AgNPs were characterized by various analytical techniques. XRD results showed four significant characteristic peaks and some unassigned peaks, these peaks might be due to the crystallization of bio-organic phase or protein on the surface of the AgNPs from *Spirulina* extract. In addition, the average size of silver crystallite was calculated from the FWHMs (full-width at half-maximum) plane of the diffraction peaks, using the Scherrer equation. The obtained average size around 18 nm, which is well matched with the TEM results these findings are in agreement with the previous studies on the crystallinity of *Spirogyra varians* mediated silver nanoparticles [45].

FTIR spectrum of the biosynthesized silver nanoparticles revealed that C–H stretching vibration mode, C=O stretch, C–H bend stretching vibration mode and C-C stretch, aromatics, are the major factor for the synthesis of silver nanoparticles. The above findings are in agreement with the earlier findings of Theivandran *et al.* [46]. In addition the strong stretching vibration of O–H functional group was shifted to lower wavelengths due to the reduction of Ag^+ ions into Ag^0. A similar result was observed in *Tribulus terrestris* mediated synthesis of silver nanoparticles [47].

Earlier finding revealed that the synthesis of silver nanoparticles using algae such as *Botryococcus braunii*, *Coelastrum* sp. and *Scenedesmus* sp. showed spherical morphology with size variation of 15.6, 19.2 and 10-15 nm, respectively [48,49]. The present study results also demonstrated that the AgNPs are well-dispersed, spherical and oval in shapes with size ranging from 2 to 18 nm. EDX analysis results proved the elemental constituent of synthesized AgNPs. It shows the major silver peaks with other minor peaks, this may be due to the capping of AgNPs by the biomolecules of *Spirulina* extract and absence

of other peaks proved the purity of silver nanoparticles. XRD and SAED diffraction pattern results clearly demonstrated that the synthesized AgNPs are polycrystalline in nature. Similar results were also observed in diatom *Amphora* sp. mediated synthesis of silver nanoparticles [49].

V. parahaemolyticus is a ubiquitous marine bacterium in coastal waters and as a virulent pathogen for marine finfish and shrimps. Controlling of this pathogenic bacterium is a major constraint for the aquaculture industry. Antibiotics and chemicals play a major role to manage this *Vibrio* group however drug resistance may be the chief constrains. On the other hand, bacteria would have to endure simultaneous mutations in every critical function within a single generation to avoid the negative effect. This problem is common with antibiotics because it chiefly targets one process, whereas silver affects a number of cellular processes as well as the membrane integrity, hence the development of resistance to silver would be very rare. In addition, during mutation the antibiotic is metabolized from the cell of bacteria, which will decrease the sensitivity to the antibiotic [50]. However, silver nanoparticles detained the biological process of bacterial cell and its multiplication. Biosynthesis of gold and silver nanoparticles has been studied using live *Spirulina* culture extract [31,51]. However, its antibacterial efficacy against aquaculture pathogen *V. parahaemolyticus* has not been investigated so far. Hence, the present study has been focused to evaluate the antibacterial efficacy of synthesized AgNPs against *V. parahaemolyticus*. The green synthesized silver nanoparticles using *Spirulina* extract showed an excellent antibacterial activity because of their size, since the smaller dimensions nanoparticle can easily penetrate into bacteria and reach the nuclear content and arrest the bacterial multiplication [40]. On the other hand, Sondi and Sondi demonstrated that the antibacterial activity of silver nanoparticles against Gram-negative bacteria were dependent on the concentration of silver nanoparticles and closely linked with the development of cavity or pore in the cell wall of bacteria [15]. This could be the reason for the most excellent activity of synthesized silver nanoparticle using *Spirulina* extract against *V. parahaemolyticus*.

Artemia is a small marine organism, its biology is more or less similar to the shrimp and moreover easy to maintain in the laboratory condition and live food for marine finfish and shrimp larval. Hence, most of the aquaculture researchers are using *Artemia* as a model organism for bacterial and toxicity studies. In this work, we are interested to know the *in vivo* antibacterial potential of *Spirulina* mediated synthesized AgNPs were tested against *V. parahaemolyticus* using *Artemia nauplii* as a model organism. The results clearly represents *Artemia nauplii* treated with *V. parahaemolyticus* alone (Group-2) showed 100 % mortality due to the virulance of the bacterium. In contrast *V. paraheamolyticus* infected *Artemia nauplii* treated with 5 µg/mL of *Spirulina* mediated silver nanoparticles (Group-4) significantly decreased ($P < 0.005$) the mortality rate when compared to Group-2. At the same time the *Artemia nauplii* treated with silver nanoparticle alone Group-3 exhibited insignificant amount of mortality and the *Artemia nauplii* survival was relatively similar to the control group which clearly indicates that the *Spirulina* mediated silver nanoparticles are non-toxic material to the *Artemia nauplii*. Although antibacterial effect of AgNPs has been extensively studied, only a few reports are available on the antibacterial effect of AgNPs against the *Vibrio* species. Vaseeharan *et al*. [39] reported that the antibacterial efficacy of AgNPs against *Vibrio* species using *Fenneropenaeus indicus*. Similarly, the antibacterial potential of *Prosopis chilensis* mediated AgNPs against *Vibrio* species using *Penaeus monodon* [52] has been reported. The above finding coincides with the present study that the green synthesized silver nanoparticles using *Spirulina* extract exhibited stupendous antibacterial efficacy against *V. parahaemolyticus* and improves the survival of *Artemia*. Hence, the present study suggests that the *Spirulina* mediated synthesized nanoparticles are nontoxic and environmentally acceptable, which can be applied to control the growth of *V. parahaemolyticus* in aquaculture system. Furthermore, larger *in vivo* studies are necessary to validate the advantages of *Spirulina* mediate synthesized AgNPs.

Conclusion

The present investigation demonstrated the prospects of nanotechnology to overcome the limitations of conventional methods practiced to control the disease outbreak in aquaculture. The synthesized silver nanoparticles using *Spirulina* extract were mostly polycrystalline in nature and spherical in shape ranged between 2 to 18 nm. The antibacterial effect of silver nanoparticles against *V. parahaemolyticus* was proportional to the concentration of nanoparticles that reflected the strong zone of inhibition around the bacterial colonies. 5 µg/mL concentration of silver nanoparticles reduced the virulence of pathogenic bacteria and maintained the survival rate of *Artemia nauplii*. The present study revealed that the application of biosynthesized silver nanoparticles using *Spirulina* extract is an effective alternative remedy for controlling vibriosis disease in aquaculture. However, the mobility of AgNPs into cells and its risk aspects in the aquaculture environment should be strengthened for intense application.

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