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Original article

Efficacy of encapsulated biogenic silver nanoparticles and its disease resistance against *Vibrio harveyi* through oral administration in *Macrobrachium rosenbergii*

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ABSTRACT

Biological synthesis of silver nanoparticles (AgNPs) by *Cheatomorpha antennia* and its *in vitro* and *in vivo* antibacterial activity against *Vibrio harveyi* in *Macrobrachium rosenbergii* was demonstrated in the study. *In vitro* growth curve analysis, cell viability and bacterial inhibitory assays were performed to test the efficacy of synthesised AgNPs against bacteria. Sodium caseinate was used as an encapsulating agent to deliver the antibacterial drugs and the commercial process of microencapsulation comprises the antibacterial bioelements for oral administration to improve the disease resistance of AgNPs against *V. harveyi* due to the eco-friendly for non-toxic behaviour of nanoparticle and their treatment. Characterisation of antibacterial silver was performed by UV spectroscopy, X-ray diffraction, Fourier Transform Infrared spectroscopy and Scanning Electron Microscopy. The peak at 420 nm showed the presence of nanoparticles in the solution and the crystal nature of the particle was identified by the XRD. FTIR characterised the functional *harveyi* biomolecules and further SEM confirmed the size of the nanoparticles around 24 ± 2.4 nm. Experimental pathogenicity of *V. harveyi* showed 100% mortality at the 120th hour. Treatment of encapsulated AgNPs was administered orally for the relative percentage of survival which acquired almost 90% of survival till 30 days of exposure. In conclusion, the microencapsulation of AgNPs in the biopolymer matrices promotes the health, growth responses, immunity and disease resistance of encapsulated AgNPs with an improved relative percentage of survival.

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1. Introduction

Shrimp aquaculture reported tremendous growth and has a vast business market for the commercial cultivation of economically essential shrimp species. Cultured shrimp farms gain a lot of attention in producing healthy supplements for consumers looking for the protein requirement. *Vibrio* species are considered one of the primary and harmful opportunistic pathogenic bacteria that cause substantial economic loss through bulk mortality in the cultured farms of *Macrobrachium* (Austin and Austin, 2007; Vaseeharan and Ramasamy, 2003). Traditionally, the treatment for the prevention and control measures of vibriosis in the cultured farm unsuccessful. The improper administrations of few commercial antibiotics failed to combat bacterial infection, which also causes resistance to the bacterial strains. Therefore, there is an urgent need to develop safe, cost-effective, resistant free antibacterial drugs to develop sustainable aquaculture. In aquaculture, there is a continuous demand the sustainable aquaculture production by introducing new, safe and effective phytocompounds, plant oils, probiotics. Thus, various natural products based antibiotics can be used for the safe administration as biocidal drugs instead commercial antibiotics to increase the growth and minimise larval mortality in the cultured farms.

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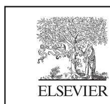

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As a recent advancement of nanotechnology, the use of nanoparticles with potential antibacterial activities based on their broad spectrum of antimicrobial activity, among various nanomaterials silver was considered as most effective against such opportunistic aquaculture pathogens (Sondi and Salopek-Sondi, 2004). Silver nanoparticles have a diverse property with the environment and human exposure; also it poses good antibacterial activity. The nanoparticles play a significant role in drug delivery systems (Park et al., 2011). Nanocrystalline silver particles have found tremendous applications in the field of high sensitivity biomolecular detection and diagnostics (Schultz et al., 2000), antimicrobials, and therapeutics. The use of environmentally benign materials like plant leaf extract (Parashar et al., 2009), bacteria (Jain et al., 2009), fungi (Bhainsa and D'souza, 2006), and enzymes (Willner et al., 2007) for the synthesis of silver nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and other biomedical applications; as they do not use toxic chemicals for the synthesis protocol. Chemical synthesis methods lead to absorption on to the surface that may have an adverse effect in medical applications. Green synthesis provides advancement over chemical and physical methods as it is cost-effective, environment-friendly, and easily scaled up for large-scale synthesis. There is no need to use high pressure, energy, temperature, and toxic chemicals in this method. Green synthesis of silver nanoparticles using seaweed is considered a novel approach because it is considered a potent beneficiary species with enormous biological functions and bioactive molecules in its nature. It helps in preventing various disorders and treatments in animals and humans. Plant secondary metabolites are had abroad spectrum of biological properties, including antibacterial, antifungal, antiviral and anti-inflammatory properties (Lindequist et al., 2001; Newman et al., 2003). This type of biologically synthesised nanoparticles helps in recovering the causes of bacterial infection and other medicinal benefits.

Chaetomorpha antennina was reported to have a excellent phtyocompounds and good antibacterial activity according to our earlier report (Thanigaivel et al., 2015). The ability of the antioxidant, antibacterial compounds and vitamin C contents presents in the *C. antennina* excerts the possibility to reduce the silver ions when interacted with silver nitrate for the synthesis of silver nanoparticles. The use of various phytocompounds such as terpenoids, flavonoids, alkaloids, phenols and volatile halogenated hydrocarbons helps preventing cell damages and prevent the diseases occurence (Thanigaivel et al., 2014). Such secondary metabolites and active molecules help in antioxidant mechanism and radical scavenging activities (Thanigaivel et al., 2015). Based on the recent surveys, engineered nanoparticle production reaches high. The most nanomaterial used for the consumer benefits was silver, as reported by (Jovanović et al., 2011; Berube et al., 2010). This novel approach of biologically synthesised nanoparticles encapsulation in the non-toxic sodium caseinate polymer mixed with commercial fish feed was done. It was used to embed the different concentration formulated AgNPs in the fish diet were given through oral administration to promote the immune system and disease resistance of prawns *M. rosenbergii* against *Vibrio harveyi.* The formulation was also given through the intramuscular and immersion routes to check the effective antibacterial activity *in vitro* and *in vivo* pathogenicity experiments, and its treatment methods have also been assessed.

2. Materials and methods

2.1. Collection and maintenance of experimental animals

Healthy prawns of freshwater *M. rosenbergii* with an average body weight of 10 to 12 gms were collected from the hatchery nearby Sirkali Nagapattinam, Tami Nadu, for experimental purpose. Healthy prawns were transported to the laboratory with controlled aeration with no record of diseases and symptoms. Animals were maintained with commercial feeds.

2.2. Physico-chemical parameters

The physico-chemical parameters such as pH, temperature and dissolved oxygen (DO), alkalinity, hardness, nitrate contents were measured following standard procedures. The dissolved oxygen was estimated by the Winkler method (Strickland and Parsons, 1972).

2.3. Preparation of seaweed extract

Chaetomorpha antennina was collected from the Gulf of Mannar, Mandapam, India. First, the seaweed was surface sterilised with running tap water to remove extraneous substances, followed by distilled water. Next, the seaweed was shade dried for 15 days and powdered. Later, the aqueous extract was prepared by dissolving 1 g of powdered seaweed in 1000 ml of sterile distilled water, then the extract was centrifuged at 6,000 rpm, and the supernatant was stored for the study.

2.4. Preliminary phytochemical screening

The powdered seaweed soaked in the distilled water to extract the phytochemicals present in the seaweeds by standard protocol (Thanigaivel et al., 2014; Harborne, 1998). The prepared plant extracts were subjected to the screening of natural bioactive compounds such as amino acids, alkaloid, flavonoids, proteins, saponin, tannin, phenolics, etc. FT-IR analysis performed to reveals the presence of characteristic functional groups present in the seaweed extract.

2.5. Preparation of bacterial culture

Vibrio harveyi strain was purchased from microbial type culture collection (MTCC) and confirmed by the Bergy's Manual of Systemic Bacteriology. The strain was further used for the experimental pathogenicity and bacterial challenge studies.

2.6. Synthesis of silver nanoparticles

1 mM Silver aqueous nitrate ($AgNO_3$) solution was prepared and used for the synthesised silver nanoparticles. 10 ml of *Chaetomorpha antennina* extract was added into 90 ml of an aqueous solution of 1 mM Silver nitrate, with different condition, temperature, and time interval was followed to optimise the process. This was performed to achieve more yield of AgNPs with less time with the continuous stirring condition for 15 mins at 20 °C to reduce $AgNO^3$ into Ag + ions and kept at room temperature. Then the product was centrifuged at 10,000 rpm for 10 mins to for the efficient separation of nanoparticles.

2.7. Characterisation of AgNPs

Characterisation of silver nanoparticles was done using various techniques like UV–Vis (Systronics 2201, India) spectrophotometer to check the absorbance of the AgNPs at a range of 200–700 nm. Dynamic Light Scattering (DLS) (90 Plus Particle Size Analyzer, Brookhaven Instruments Corporation, Holtsville, USA) was employed to check the particle size and distribution at the colloidal surface, and its zeta potential was also studied to check their long term stability. Scanning electron microscopic (SEM) technique used to measure the size and presence of silver nanoparticle with

the nanometer scale. FT-IT spectroscopy with the operating resolution of 1.0 cm^{-1} by Perkin Elmer Spectrum with KBr press and Mylar beam splitter was done to screen the presence of functional groups involved in reducing metal ions; XRD analysis was also performed to study the nature of metal irons and their dimension of particles.

2.8. Encapsulation of AgNPs in the polymer matrix

The preparation of sodium caseinate was prepared in the distilled water. The synthesised nanoparticle was added in different concentrations, and the solution was kept under the constant stirring condition to avoid agglomeration. A polymer containing nanoparticles was added dropwise in the methanol solution containing 1% (w/v) of glutaraldehyde as a cross-linking agent, and 0.1% of concentrated HCL was used as a catalyst. The microbeads were prepared using 25 ml of 1 mm hypodermal syringe. Under the constant stirring condition, the beads were formed in methanol solution. The prepared beads were air-dried and kept under room temperature overnight (Quaroni and Chumanov, 1999).

2.9. Comparative antibacterial study

The disk and well diffusion methods were followed for the antibiotic sensitivity assessment (Thomas et al., 2013; Thanigaivel et al., 2015). The antibiotic disc was obtained from Hi-Media. The antibiotic disc used as tetracycline with 20 mg/ml concentration was used. After making a lawn culture of the bacterium, the antimicrobial susceptibility disc was carefully dropped onto the surface of the Muller Hinton agar in the plate using aseptic techniques. The extract loaded disc and sliver nanoparticle loaded disc were applied to the lawn culture of the test organism. The two discs were placed 24 mm apart from each other to avoid overlapping the zone of inhibition. Each disc was pressed down firmly onto the base of the agar plate using sterile forceps. The plates were incubated at 30 °C for 24 h for a clear circular area (Zone of Inhibition) in the bacterial lawn around the antibiotic disc. The size of the zone of inhibition was measured. The test was carried out in triplicates.

2.10. Growth kinetics study

The potentiality of silver nanoparticles (AgNPs) and their antibacterial activity was confirmed by the time to kill assay, which showed maximum antibacterial activity against a bacterial pathogen. The bacterial inoculum of Vibrio harveyi 100 μl was supplemented with 5, 10, 15, 20 and 30 and 50 μg of AgNPs cm-3. Negative control was maintained without the nanoparticle. The growth of the bacterial cells was monitored at every 1-hour interval by measuring the optical density at 600 nm by using a spectrophotometer. Optical density of 0.1 corresponds to the concentration of 107 cells cm-3 (Vaseeharan et al., 2010).

2.11. Experimental pathogenicity in healthy prawns

2.11.1. Infection via intramuscular injection

The adult prawns at the rate of five per tank were maintained in 100- l capacity fibreglass tanks. Regular sanitisation was taken care of, water provided with good aeration. The animals were fed with commercial pelleted feed (CP feed, Thailand). Different concentrations (10^3, 10^4, 10^5, 10^6, and 10^7 CFU $animal^{-1}$) of bacterial suspension were used for this experiment. For adults prawn, 20 μl of bacterial suspension from each of these five concentrations was injected intramuscularly (IM) using 1 ml insulin syringes in the third abdominal segment. Control Prawn received only sterile saline (Ducklow et al., 1980; Egidius, 1987; Thanigaivel et al., 2015).

2.11.2. Experimental infection by immersion

Healthy prawns were reared in aquarium tanks of 100-l capacity containing sterilised freshwater with continuous aeration. Air stones and air tubes were sterilised by immersing them in 2.6% sodium hypochlorite and washing them thoroughly with tap water before use. Aseptic techniques were used throughout the experiment. The prawns were exposed to different concentrations of bacterial cells (10^3, 10^4, 10^5, 10^6 and 10^7 CFU ml^{-1}). The control comprises freshwater fishes. The experiment was conducted in triplicates in each bacterial concentration. Animals were monitored for clinical signs of disease and mortality (Thanigaivel et al., 2015).

2.12. Fish feed preparation for oral administration

Commercial shrimp feed procured from the local vendor, and it was mixed into distilled water. Then the soaked feeds were ground well and made into a fine paste. It was then subsequently added into the nanoparticle-containing microbeads for the homogenous coating by the mixing process. The amount of feed provided for the mixing will be 1% of the body weight. The amount of microbead used for the mixing of fish feed is approximately calculated to make sure the Prawn receive the 0.1% of the nanomaterial and sodium caseinate to improve the growth performance of the body, immunity and disease resistance. Throughout the experiment, the Prawns were monitored regularly to ensure the uptake of fish beads for growth and development. The entrapment efficiency was tested by calculating the ratio between the initial mass of nanoparticles to be encapsulated into the final mass of the product.

2.13. SEM analysis of nanoparticles encapsulated bead

Scanning Electron Microscopic (SEM) analysis was performed to study the microencapsulated bead's external surface and internal structure. It will be done using a Hitachi S-4500 SEM machine. First, thin films of the sample were prepared on a carbon-coated copper grid by just dropping a minimal amount of the sample on the grid, the extra solution was removed using a blotting paper, and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

2.14. FT-IR analysis

FT-IR analysis was performed to ascertain the functional groups responsible for the interaction between the aqueous solution of seaweed extracts and AgNPs and the functional groups responsible for reducing the Ag^+ metal ions in the formation of silver nanoparticles. This was carried out with the Perkin Elmer FTIR Spectrum, instrumented at the resolution of 1.0 cm^{-1} with KBr press and Mylar beam splitter.

2.15. Biosafety profile of AgNPs for the in vivo treatment

The comparative biosafety profile of biologically synthesised AgNPs was tested against *V. harveyi* by oral administration, intramuscular and immersion methods to control the disease-causing pathogens against prawns affected by the pathogens as *V. harveyi*. The *in vivo* treatment efficacy of AgNPs tested for their optimum exposure time to the toxicity bioassay. The assay was carried by varying the synthesised nanoparticles concentration ranges from 20, 50, 100, 150, 200, 250 to 500 mg/l administered through Immersion routes by challenging it against bacterial inoculums intramuscular infection study 20–30 μl of nanoparticles given intramuscularly and challenged against *V. harveyi*. Time taken for the optimum exposure of AgNPs challenged against the causative

organisms tested for the relative percentage of survival, and the toxicity behaviour prawns were carried out.

2.16. In vivo antibacterial assessment of encapsulated AgNPS through oral administration

Experimental animals were grouped into the test, control for the pathogenicity challenge. Test animals were categorised into Test 1 and Test 2. Control groups were fed only with commercial fish feeds. Test 1 groups were allowed only with the sodium caseinate microbead. Test 2 considered with nanoparticles encapsulated microbead for the treatment. Each group is divided into 15 prawns per tank with required aeration and diet control before the experimentation. The experiment was conducted in triplicate.

2.17. Cell viability assay

Trypan blue staining was followed by suspending the cells into the serum-free media and adding the desired concentration of seaweed extract with the bacterial cells with the 1×10^6 cells (Thangaraj, 2016). Then the assay mixture was made up to 1 ml using PBS and incubated at 37 °C for 3 h; further, the addition of 0.1 ml of cell suspension with 0.1 ml of 0.4% typan blue was added and loaded onto the haemocytometer; finally, the number of live and dead cells was calculated using the formula.

$$\% \text{ Viablity} = \text{number of viablecells}/\text{Number of total cells} \times 100$$

2.18. Histopathological investigation

Histopathological investigation of organs was studied for the internal organ damages caused by the bacterial pathogen, and its treatment by AgNPs was analysed. The organs such as gills, tissues, hepatopancreas were dissected out from control fish, infected fish, and treated fish and studied for the level of organ damages caused by the bacterial pathogen compared to the treatment groups (Thanigaivel et al., 2014).

2.19. Statistical analysis

In all the experiments, the significance of differences in the mortalities observed among prawn groups was assessed by the chi-square test. Probabilities lower than 0.01 ($P < 0.01$) were considered significant.

3. Results

3.1. Collection and maintenance of experimental animals

Healthy experimental prawns were obtained from the hatchery, and seaweeds collected from the coastal area were processed to synthesise AgNPs. The reduction of silver was done by biological method without the aid of any reducing agent. The phyto components and available in the *C. antennina* helped in the conversion of Ag + silver ions.

3.2. Physico-chemical parameters

Studying the quality parameters such as physiochemical parameters such as water quality, maintaining nitrogen content, dissolved oxygen, total dissolved solids, and salinity are important parameters to be considered for the successful aquaculture rearing process. Culturing farms fishes, rearing cultured prawns requires proper acclimatisation conditions for experimental pathogenicity and treatment process. Therefore, these parameters mentioned above were made sure to be at the optimum/prescribed level.

Preliminary physiochemical parameters analysis was performed on the seaweeds extracts before processing them for the synthesis of AgNPs. The presence of such phytocompounds in the seaweeds extracts will highly help to identify various active constituents. These bioactive compounds are proven to benefit various health care. Its biomedical applications were proven to be an excellent source for good antioxidant, anti-inflammatory, anti-fungal, anti-malarial, anti-microbial, and anti-diabetic and many health care applications to prevent various disease and disorders.

3.3. Biogenic silver nanoparticles synthesis

Preparation of nanoparticle was done using the biological reduction method using seaweed extracts, phytocompounds present in the seaweed extracts were helped in converting $AgNO_3$ into AgNPs under controlled condition. Bioactive compounds such as tannins, alkaloids, phenols, steroids, flavonoids, proteins, and amino acids help reduce the $AgNO_3$ to Ag^+ ions; active compounds containing vitamin C contents were found to be responsible for the conversion of AgNPs. During the reaction, the time solution containing seaweed extract in silver solution turned into a brown colour after incubation. The control solution was kept blank without adding the silver nitrate for the reduction, and it remains unchanged in colour after the overnight incubation. The colour intensity of the silver nanoparticle solution was further confirmed with the peak indication at different wavelength starts from 300 to 700 nm. The peak absorption of AgNPs was found to be and confirmed at 420 nm with a dominant peak. After converting nanoparticle size, shape and dimensions play a crucial role in the *in vitro* and *in vivo* treatment process.

3.4. Characterisation of AgNPs

Characterising the nanoparticles is essential to confirm the particle behaviour, structural, functional properties, and nanoparticles. Primary confirmatory analysis of nanoparticle will be done using Ultra visible spectroscopy. UV–Visible spectroscopy confirms the synthesis and formation of nanoparticles by the absorbance peak at 420 nm, as shown in Fig. 1A. The subsequent characterisation technique is X-ray diffraction analysis and is done by XRD techniques. XRD pattern was used to determine the nature of the sample. It determines the property of the nanoparticles, whether the particle exists in crystalline, amorphous or any other form, by predicting the two theta value ranges from 20 to 80. In addition, the XRD pattern had revealed the intense peaks at (111, 222, 300 and 411). These strongly reflect the Bragg reflections with face-centred cubic (fcc) confirming silver in the sample as shown in Fig. 1B.

Then the DLS method was performed to show the average particle and size and the stability of the particles over the agglomeration process. The DLS method was shown in Fig. 1C with a mean particle size average of 184. In addition, 4 nm and the zeta potential were around −21.4, indicating higher particle stability. Another essential characteristic of nanoparticle confirmation is done by Scanning Electron microscopes (SEM). The SEM micrograph clearly shows silver nanoparticle formation in the liquid silver NPs suspension provided for the analysis. Here the results are shown as mostly the spherical shaped AgNPs with varied size range. The size range of the silver nanoparticles depicted in the SEM micrograph was found to be around 20–85, with a cumulative particle size range of 24 ± 2.4 nm in range, as shown in Fig. 1D.

Then the synthesised AgNPs were tested by Fourier infrared spectroscopy (FTIR). Different functional groups were exhibited after the FTIR analysis, which confirms the vital amino, nitro and

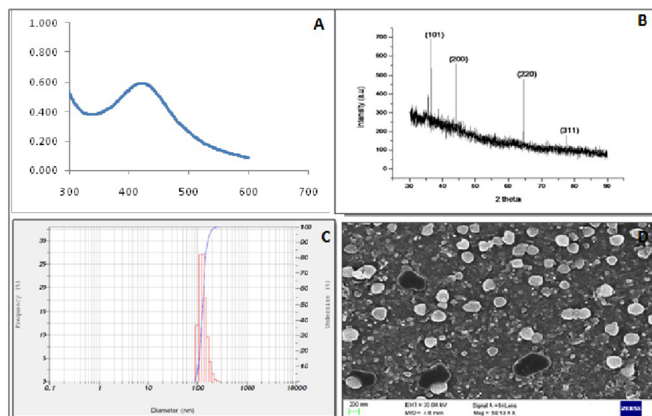


Fig. 1. (A). UV–Visible spectrum of silver nanoparticles peak at 420 nm exhibit the confirmation of silver nanoparticles in the suspension (B) XRD pattern of AgNPs confirmation of crystal nature (C) DLS confirms the particle size of the AgNPs (D) SEM image of AgNPs with spherical shape particles.

aromatic ring were found to present in the sample AgNPs sample mixture which seaweed extract has interacted with AgNPs and un-interacted with AgNPs. The functional groups were presented in Fig. 2.

3.5. Comparative antibacterial study

In vitro antibacterial assessment of silver nanoparticles was tested against the *Vibrio* pathogen. *V. harveyi* was the potent pathogen that affects and cause huge mortality in the majority of the farmed animals, especially freshwater prawns and seawater shrimps. Comparative antibacterial activity against *Vibrio* pathogen was tested by disc and well diffusion methods showed the zone of inhibition at 20 ± 1 mm and 17.8 ± 2 respectively. In contrast to this, the control plate does not have any antibacterial activity and zone of inhibition since it was kept culture lawn and applied only silver salt solution. Due to the trace activity of antibacterial silver, less than 1 mm of the zone of inhibition has occurred in the control plate. The detailed efficacy of AgNPs was tested using the bacterial growth inhibitory assay as presented in Fig. 3.

Growth kinetics study bacterial growth kinetics of *V. harveyi* was performed for overnight culture. Different concentration AgNPs was added in different concentration of bacterial colonies tested. Bacterial dilution ranging from 3×10^4 CFU/ml was tested. Gradual reduction of bacterial colonies was observed, and complete growths of the bacterial colonies were arrested at the concentration of 30 μg of AgNPs, as shown in Fig. 4.

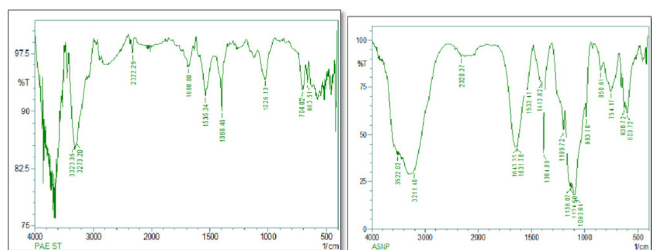


Fig. 2. FT-IR Spectrum (A) Plant algal extract (B) Silver interacted seaweed extracts.

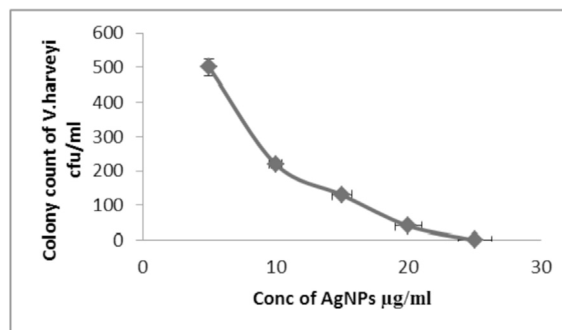


Fig. 3. Anti-bacterial assessment and bacterial inhibitory assay of *V. harveyi* against AgNPs. Concentration from 10 to 30 μl was used to check the amount of viable cells of *V. harveyi.*

3.6. Experimental pathogenicity in healthy prawns

In vitro efficacy of the AgNPs was tested in the plate methods and bacterial growth curve method to test the efficacy of AgNPs by *in vivo* method. A pathogenicity experiment was conducted to test the mortality rates, and the virulence of bacterial colonies was tested by intramuscular (IM) and immersion methods. The administration of bacterial dose administration carries out intramuscular injection through the dorsal region of prawns to create experimental pathogenicity. The dosage optimisation for IM infection 20–25 μl of bacterial culture with the CFU of approximately 10^6 CFU/ml. The immersion method was carried to disperse the bacterial culture in the medium where prawns were acclimated. In the liquid medium, 50–100 μl of culture were added into the 25-litre water-containing tank luminescence effects in all the infected prawns until the 6th days of infection. The result of IM and Immersion based pathogenicity was shown in Fig. 5. The biosafety profile of the silver nanoparticle was also tested by cross challenging the prawns against bacterial infection through intramuscular and immersion method. The *in vitro* effective concentration of silver nanoparticle was given through the same route to check the *in vivo* efficacy in the experimentally infected groups through challenge study.

Encapsulated feed for the treatment of *Vibrio harveyi* was tested in a different ratio. A biological medicated Silver nanoparticle was encapsulated in the Sodium caseinate is for oral administration. Different test groups were prepared to evaluate the efficacy AgNPs encapsulated feed. The survival rate of prawns was tested after 30 days. A challenging bacterial study was conducted in the test groups to enhance the disease resistance activity of antibacterial

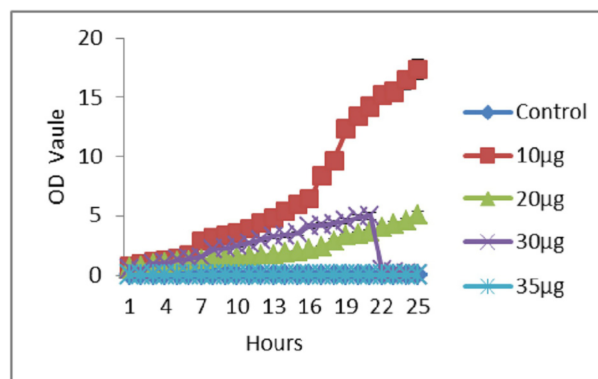


Fig. 4. Growth curve analysis of *V. harveyi* interacted with different concentration of AgNPs.

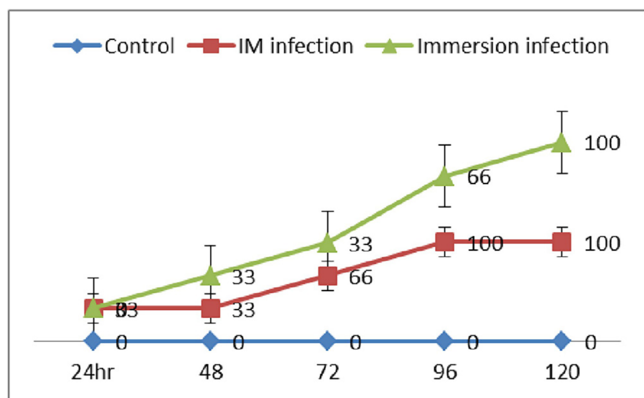


Fig. 5. Experimental Pathogenicity of *V. harveyi* by Intramuscualr (IM) and Immersion methods was performed by triplicate.

silver feeds. Test group A was fed with sodium caseinate challenged with *V. harveyi*. Test group B was challenged with silver nanoparticles encapsulated with sodium caseinate coated with commercial fish feed. The survival percentage was comparatively increased in test group B compared to test group A challenged with bacteria. The percentage of survival and disease resistance was recorded as above 90% in test group 2 until the 22nd day of the experiment. At the end of the 30th day, the relative percentage of survival (RPS) was maintained 86% on the 30th day. Test group

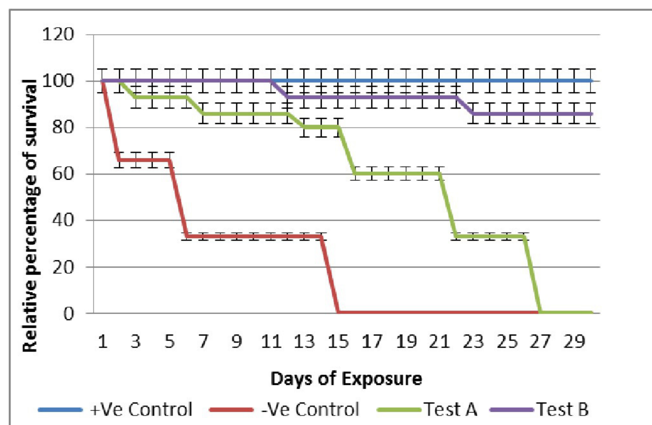


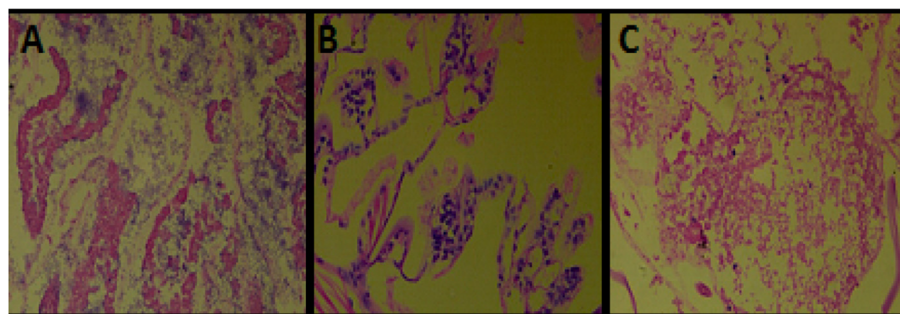
Fig. 6. Encapsulated feed treatment and percentage of survival was observed. Positive control only with saline, negative chanllenged with bacteria, test A given sodium caseinate with bacteria challenged, test B was AgNPs challenged with bacteria.

1 showed a maximum of 20% of the survival and failed to maintain RPS throughout the experiment. The control group administered only with commercial fish feed challenged with bacterial maintain 0% survival and achieved 100% mortality on the 15th day of the experiment. Positive control only with commercial saline, negative control administered with bacteria and challenged with commericla feed. The graphical representation of the survival rate is clearly shown in Fig. 6. Biopolymers are one of the essential sources of protein-rich vitamins and fibres. The encapsulation of such antibacterial drugs in the biopolymers such as sodium caseinate will inhibit the microbial growth when fed with a fish diet and improve the oral bioavailability and biodistribution when it reaches the system.

3.7. Cell viability assay

Cell viability assay was performed to assess the *in vitro* efficacy of the silver nanoparticles against the bacterial culture. Different concentration of bacterial culture and anf AgNPs were mixed in the suspension to monitor the growth characteristics of bacterial culture against silver nanoparticles. 2 ml, 5 ml and 10 ml of a broth culture of *V. harveyi* were prepared, and the concentration of 50, 100 and 200 μl of AgNPs are added in the culture with strong bactericidal activity. After 24 h of incubation, optical density was measured to ascertain the viability of bacteria and the efficacy of AgNPs in killing the bacterial colonies; after completing the test, AgNPs were found to have a good capacity anti-proliferative activity and good potential to inhibit the growth of *Vibro.*

The histopathological investigation was performed to study the tissue sections of experimental prawns to determine the internal damages caused by the pathogenic effects of *V. harveyi during* the treatment with silver nanoparticles. Due to the pathogenic potential of bacteria, essential organs such as gills, intestine, brain are slightly affected by necrosis and cell damages when challenged with AgNPs; the levels of damages and internal structural changes were clearly shown in Fig. 7. These structural changes were observed in the naturally infected and experimentally infected prawns to the control prawns that were not infected by any pathogens and external/internal stressors. The portions of gill lamella are mainly affected by the virulence bacterial infections, and infected prawn gill lamella will exhibit shrunken nuclei with extensive vacuolation, as shown in Fig. 7A. The next predominant organ affected by the pathogen is the hepatopancreas. Many of the adult infected prawns shows vacuolation in all types of epithelium cells. All four tissues are thickened, especially at the junction of the connective tissues as shown in Fig. 7B. In heart cells, the endothelial cell seemed to be swollen and vacuolated when infected; hemocyte infiltration was also observed in the cardiac muscles upon the infection, as shown in Fig. 7C.



|Gills **(B) Hepatopancreas cells** **(C) Heart cells**

Fig. 7. Histopathological examination of *V. harvey*i infected cells challenged with AgNPs infected cells.

4. Discussion

The application of silver nanoparticles gained a lot of attention in the broader area because of its multifunctional abilities and targeted specific actions. These AgNPS are well known for the antibacterial properties, and many different sectors have commonly utilised such properties. Now the boom of AgNPs creates a trend in aquaculture sectors, the commercial silver application in aquaculture have been increased to fight against a broad spectrum of disease-causing pathogens (Morones et al., 2005; Vaseeharan et al., 2010). Especially in the intensive and semi-intensive farms for the commercial cultivation of shrimps, prawns, and other types of fishes have been increased due to the increasing demands; however, the chances of disease-causing pathogens and predator organisms were also emerged to hinder the growth of fishes and cause colossal mortality. This kind of problems may lead to severe economic loss in the aquaculture business. The synthesis of silver salts using various organic and inorganic sources has been reported in recent days to improve the biopotential of the silver nanoparticles. The reducing agent of silver is identified from the various plant origins. Such phytocompounds helps in synthesising the various metallic nanoparticles (Shankar et al., 2003; Shankar et al., 2004) Recently (Vaseeharan et al., 2010) had reported the synthesis of silver nanoparticle using green tea leaf extract to control the pathogenic virulence of *vibrio*. This present study investigates the synthesis of silver nanoparticles and their application towards vibrio pathogen to enhance the disease resistance in freshwater prawns. The silver synthesis in this paper was carried out by the green seaweed *cheatomorpha antennia,* macroalgae rich in various phytoconstituents, which highly help in antioxidant and radical scavenging properties. Many researchers have focused on the commercial benefits of herbs, medicinal plants and other polyherbal combinations to synthesis silver and other metallic nanoparticles to enhance the efficacy of the biologically synthesised silver nanoparticle in different sectors for various applications (Chandran et al., 2006; Ankamwar et al., 2005; Gardea-Torresdey et al., 2003; Gibbins, 2003). Many of them were used the benefits of silver nanoparticle in commercial aquaculture applications (Kandasamy et al., 2013; Ochoa-Meza et al., 2019; Baskaralingam et al., 2012; Robertson et al., 1998; Antony et al., 2013).

The present study successfully exhibited the silver nanoparticle synthesis, which was mediated by the green seaweed extract; after adding seaweed extracts into the silver salt-containing solution, the reduction of Ag salt was achieved. Due to the excitation of silver ions the surface plasmon effect was observed, the silver nanoparticle formation was confirmed. The preliminary confirmation was done by the colour change in the solution followed by the UV visible spectroscopic technique with the absorption maxima at 420 nm to control *Vibrio* infection in freshwater prawns. This preliminary information's opens the possibility to conduct the study further, and studies reported the synthesis of silver nanoparticles with plasma excitation at 420 nm (Antony et al., 2011; Sivalingam et al., 2012; Mulvaney, 1996). The following technique was performed to confirm the nature of the AgNPs since the nature of the samples determines the efficiency in the application. The XRD pattern of the sample identified the exact nature of the silver nanoparticle. The XRD spectrum exhibited the significant peaks which ultimately confirm the presence of silver in nanoparticles in the sample; the intense peaks are shown as in the Fig. 1B are (1 1 1), (2 2 2), (3 0 0) and (4 1 1); these peaks confirm the presence of silver nanocrystals according to the Braggs reflections as mentioned by researchers (Kandasamy et al., 2013; Lu et al., 2003). Thus, it is proved that AgNPs are formed by the extracellular reduction of silver ions by the seaweed extract of *C. antennia* was in the crystalline structure.

The FT-IR spectrum was carried out to identify the type of biomolecules possibly involved in capping the formation and stabilisation of metal nanoparticles. This gives information about the type of functional elements present in the seaweed extract and biomolecules responsible for the formations of silver nanoparticles. The FT-IR spectra of silver nanoparticle synthesised by this study showed in the Fig. 2 and the peaks exhibited at different region responsible for the different groups. The present study exhibit peaks at the range of 660–704 responsible for C—H bending, 1026 to 1094 is C—O—C, stretching, 1384–1413 showed C—O stretching, 1535–1643 for C=C (Carboxylate anion and amide I), 1680–1700 responsible for C=C stretching, 3323 to 3522 is C—H stretching as described by the authors (Antony et al., 2011). Exhibited peaks at 663, 1199, 1396, 1680, 3323 correspond to a dominant peak for alcoholic and phenolic groups, indicating possibly polyphenolic encapsulation. The peak around 2322 cm^{-1} is be attributed to a peak for metal reduction. Peaks at the range of 1637, 1680 cm^{-1} and 754 cm^{-1} may correspond to primary amines correlating to proteins. The present study results reported having the terpenoids, flavonoids, alkaloids, and phenols absorbed on the surface of the metal nanoparticles. Similarly, the possible interaction of terpenoids and flavanoids for the metal nanoparticle formation with possible stretches of C—O, C—C and carboxylic groups reported in previous studies (Gibbins, 2003; Kandasamy et al., 2013).

The size and structure of the nanomaterial have been studied using a scanning electron microscope attributed particle size of AgNPs was analysed by the SEM with the mean size range of 24 ± 2.4 nm with spherical shape. The particle size range was found to be increased with increasing wavelength. Hence, the particle size was so tiny and spherical particle that it facilitated bioactive compound encapsulation for the delivery purpose. The DLS method also predicted the average particle size in the suspension. The Zeta potential of the sample was around −21.4, indicating the highly stable nature of the silver nanoparticles; zeta potential helps the particle from the aggregation by preventing the particles from moving toward the vicinity of zero; hence the negative charge of the particle facilitate the particles become more stable as shown in the Fig. 4. The average particle size distribution also calculated the particle size of the particle; it was 184.5 nm. The DLS is considered one of the compelling methods to determine the protein-bound nanoparticles and it can detect smaller amounts of particles formed during the agglomeration than the previous studies report (Antony et al., 2013; Suriyakalaa et al., 2013). Bacterial growth inhibition was determined by the bacterial growth kinetic study. It was conducted by the addition of antibacterial silver nanoparticle into the pure bacterial culture containing the colony count of approximately 3×10^4 CFU/ml overnight. Due to the inhibitory and anti-bacterial potential of AgNPs, the overnight bacterial culture growth was arrested by continuous-time interval up to 24 h; every one hour, culture growth was determined by the colorimetric methods by optical density of the culture at the absorbance at 600 nm. After the 24 h of incubation and OD values at every one hour, the graph was plotted against the time, and cell concentration was described by the method of (Vaseeharan et al., 2010; Robertson et al., 1998; Palanisamy et al., 2017), and the control sample was just kept as PBS saline of the reference. Similarly, the cell viability of the bacterial culture was determined for the no of viable cells in the culture, different concentrations of bacterial colonies were dissolved in the nutrient broth, and the varying concentrations of AgNPs were also added to check the number of viable cells in the culture. Modified method of bacterial viability assay was performed and adapted the use of the chitosan selenium to prove the disease resistance effect against the zebrafish, and it is considered as one of the best and recent method to evaluate cell viability (Xia et al., 2019).

Based on the available literature and results obtained from the *in vitro* assessment and analysis, we could conduct an *in vivo* test with silver nanoparticles to control the bacterial infection in the infected prawns. This study also provides an ample solution for the preventive measures against various *Vibrio* infections. *In vitro* results obtained from the growth of *V. harveyi* with different concentration of AgNPs showed 30 $\mu g/cm^2$ inhibitory response and effective decease in the cell count was observed. Further AgNPs controlled the cell multiplication by 7 to 8% when compared to control. The present study also suggested the increasing concentration of the AgNPs is directly related to the reduction of colony-forming units *V. harveyi* in agar plate as described by the method of Vaseeharan et al. (2010), Pal et al. (2007) and Morones et al. (2005) and our results are analogous to their results published. Keeping all the above mentioned *in vitro* results, we have conducted an *in vivo* treatment method against *V. harveyi* infected prawns for the experimental purpose. The experimental pathogenicity was conducted in three different routes to initiate the infections in the healthy adult prawns; these experimental groups were observed for seven days to achieve bacterial virulence followed by mortality. Similarly, the infected groups were challenged with the AgNPs encapsulated feed for the treatment to ensure disease resistance and biosafety efficacy. After the 30 days of post-infection and treatment, we could achieve the effective percentage of survival due to the diluted sodium caseinate-based AgNPs encapsulated feed for the treatment purpose. Similarly, the result (Kandasamy et al., 2013; Thanigaivel et al., 2014; Vaseeharan et al., 2010; Sondi and Salopek-Sondi, 2004) reported AgNPs against the various pathogenic microorganisms to check the bactericidal activity. The bacterial growth kinetics and inhibitory effects also seemed to be similar to that of the efficacy we obtained through our study. The histopathological investigation has been done to check the bacterial virulence in the infected prawns and treated prawns to assess the level of damages in the gill lamella, hepatopancreas and heart cell.

The comparative analysis was performed based on the suggested study of (Thanigaivel et al., 2014; Antony et al., 2013; Thanigaivel et al., 2015), finally, as an outcome of the study, we propose silver nanoparticles are the best anti-bacterial agents which act effectively against the various *Vibrio* pathogens, especially against the *V. harveyi.* Furthermore, the efficacy of the AgNPs was boosted up by mixing them or encapsulating them in the polymer mixture to avoid the elevated silver toxicity in the aquatic animals (Thanigaivel et al., 2021). Therefore, based on this study, we suggest a minute or moderate amount of nanomaterial in the aquafeed may improve the growth, body response and disease resistance in the aquaculture operations without causing any harm or metal toxicity to healthy and infected prawns when administered for oral treatment. This gives a partial idea about the safest administration of metal nanoparticles for lowering the accumulation of silver concentration in the prawns since the minimal concentrations of silver nanoparticles are mixed in the fish feed and the polymer matrix, which ultimately lower the toxicity and accumulation of silver nanoparticles. However, the detailed mechanism and mode of action against the disease resistance genes and animal growth parameters to be further evaluated.

5. Conclusion

The present study suggested various aspects in the oral administration of silver nanoparticles against *Vibro* pathogens which cause massive mortality in intensive and semi-intensive farms. However, considering the environmentally safest method to use on the aquaculture systems, silver nanoparticles may be recommended in the encapsulated form to promote the growth and disease responses in aquaculture organism. In conclusion, AgNps based diet administration through a biopolymer medium showed improved efficacy in controlling the *V. harveyi.*

Ethical approval

The use of fishes in the study requires no ethical clearance.

Declaration of Competing 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.

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