

Synthesis of silver nanoparticles by coastal plant *Prosopis chilensis* (L.) and their efficacy in controlling vibriosis in shrimp *Penaeus monodon*

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Abstract The present work investigated the effect of leaf extract from coastal plant *Prosopis chilensis* on synthesis of silver nanoparticles using AgNO_3 as a substrate and to find their antibacterial potential on pathogenic *Vibrio* species in the shrimp, *Penaeus monodon*. The leaf extract could be able to produce silver nanoparticles, as evident by gradual change in colour of the reaction mixture consisted of the extract and 1 mM AgNO_3 to dark brown. The silver nanoparticles exhibited 2θ values corresponding to the presence of silver nanocrystal, as evident by X-ray diffraction spectrum. The peaks corresponding to flavanones and terpenoids were found to be stabilizing agents of the nanoparticles, as revealed by Fourier transform infrared spectroscopy. The size of silver nanoparticles ranged from 5 to 25 nm with an average of 11.3 ± 2.1 nm and was mostly of spherical in shape, as confirmed by transmission electron microscopy. The silver nanoparticles were found to inhibit *Vibrio* pathogens viz., *Vibrio cholerae*, *V. harveyi*, and *V. parahaemolyticus* and this antibacterial effect was better than that of leaf extract, as proved by disc diffusion assay. The nanoparticles were then tested in the shrimp *Penaeus monodon* challenged with the four species of *Vibrio* pathogens for 30 days. The shrimps fed with silver nanoparticles exhibited higher survival, associated with immunomodulation in terms of higher haemocyte counts, phenoloxidase and antibacterial activities of haemolymph of *P. monodon* which is on par with that of control. Thus, the present study proved the possibility of

using silver nanoparticles produced by coastal *Prosopis chilensis* as antibacterial agent in controlling vibriosis.

Keywords Silver nanoparticles · Shrimp aquaculture · Vibriosis · *Prosopis chilensis* · *Penaeus monodon*

Introduction

Aquaculture is an important economic activity all over the world. In 1970, aquaculture formed 3.9% of the total global production by weight which increased to 27.1% in 2000. In 2004, aquaculture formed almost a one-third of the global production by weight, which is 32.4% (FAO 2007). About 47% of consumed fish and other seafood originate from aquaculture (FAO 2009). Aquaculture production has been increasing at an average rate of 3.9% in developed countries while at an average of 8.2% in developing countries (FAO 2007). In Asia, shrimp production is predominantly focused on the black tiger prawn, *Penaeus monodon* due to its excellent taste, universal appeal and high market and export market value. The intensified farming of *P. monodon* throughout the world has led to the outbreak of diseases which impede the aquaculture industry in many countries (FAO 2010).

Vibriosis is one of the major diseases caused in the aquatic organisms especially in shrimp in all seasons and can cause seafood-borne gastroenteritis in humans (Sujeewa et al. 2009). The term vibriosis refers to diseases associated with bacteria of the genus *Vibrio*. They are the natural inhabitants of estuarine and marine environments, well known for causing vibriosis in fish worldwide (Schäperclaus 1986). The prevention of vibriosis is a major challenge to aquafarming. There are various measures to prevent the entry of pathogenic microbes. However, techniques like sterilization to get rid of adherent microflora

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disturb the balance of microbial communities and favour exponential growth of opportunistic bacteria (Baticados et al. 1990). The usage of antibiotics is another option, but the indiscriminate use of antibiotics has led to an increased number of antibacterial-resistant bacteria, and ultimately compromises the treatment of bacterial infections in humans (Gersema and Helling 1986; McDermott et al. 2002). The emergence of multi-resistant bacteria to antibacterial drugs has increased the need for new alternatives against microbial pathogens (Tollefson and Miller 2000).

Prosopis chilensis is a small, deciduous, usually spiny, freely branched tree found commonly in the arid and semi-arid regions. They possess the ability to grow in seawater salinity. The tree is drought hardy and it regenerates rapidly. Hence it has been observed as common weed, coming up singly and in groups in various habitats. The species is indigenous to the Pacific coast of Peru, Bolivia, Chile, and eastern Argentina. The pods, beans and leaves are browsed and used as fodder, whereas the wood is used as fuel in many regions of the world. The sweet pods are eaten or used to make a drink. The tree is also planted for shade and thornless varieties are used as ornamentals. They are also known as a candidate for erosion control and soil stabilization in arid lands, due to its deep rooting habit (Orwa et al. 2009). The ability of this plant to withstand the extreme climatic condition might be due to the presence unique biological compounds. This assumption prompted us to select this particular plant as our experimental plant for silver nanoparticle synthesis.

Silver nanoparticles have been tested in various fields of biological sciences viz. drug delivery, wound treatment, binding with HIV gp120 protein (Elchiguerra et al. 2005). It is well known that silver ion and silver-based compounds are highly toxic to microorganisms (Slawson et al. 1992) and they are used as antibacterial compounds (Knetsch and Koole 2011; Jain and Pradeep 2005; Son et al. 2004; Li et al. 2005; Baker et al. 2005; Shrivastava et al. 2007; Pal et al. 2007; Kathiresan et al. 2010). Use of plants for synthesis of nanoparticles could be advantageous over other environmentally benign biological processes as this eliminates the elaborate process of maintaining microbial cell cultures. In this context, the present investigation was designed to synthesise silver nanoparticles using coastal plant *Prosopis chilensis*, and to evaluate antivibrio activity of the nanoparticles against shrimp pathogens.

Materials and methods

Chemicals

All analytical reagents and media components were purchased from Hi-media (Mumbai, India) and Sigma chemicals (St. Louis, USA).

Synthesis of silver nanoparticle

Leaf extract was made from *Prosopis chilensis* L. (Fabaceae) collected from Parangipettai (Lat.11°29'N; Long. 79°46'E) in the south east coast of India. Fresh leaves weighing 20 g were washed thoroughly with distilled water, extracted in 100 mL of boiling distilled water for 5 min. For reduction of Ag⁺ ions, 5 mL of leaf broth was added to 45 ml of 10⁻³ M aqueous AgNO₃ solution. The reduction of Ag⁺ ions was monitored by visual observation and measuring the absorbance of the solution in a range of wavelengths ranging from 300 to 700 using UV-visible spectrophotometer (Elico, Chennai).

Characterization of silver nanoparticles

The X-ray diffraction (XRD) measurement of silver nanoparticles synthesized by leaf extract was carried out using Cu-K α radiation source in powder diffractometer (PANalytical X'per PRO model X-ray diffractometer), on films of the solutions drop coated onto glass substrate on the instrument operating at a voltage of 50 kV and a current of 30 mA.

For Fourier Transform Infrared spectroscopy (FTIR) measurement, the following method was adopted. The silver nanoparticles were synthesized after 24 h of reaction with leaf extract, centrifuged at 10,000 rpm for 15 min, and the pellet was redispersed in sterile distilled water to get rid of any uncoordinated biological molecules. The centrifugation and redispersion in sterile distilled water was repeated three times to ensure better separation of free entities from the metal nanoparticles. The purified pellets were then dried and the powder was subjected to FTIR spectroscopy measurement (Paragon 500, Perkin Elmer-RX1 spectrophotometer) in the diffuse reflectance mode at a resolution of 4 cm⁻¹ in KBr pellets.

After 24 h of incubation, the reaction mixture was used to form a film of silver nanoparticles on carbon-coated copper TEM grids (Electron microscopy sciences, Hatfield, PA 19440) and analyzed under a transmission electron microscope (JOEL, JEM 100 SX) at a voltage of 120 kV.

Antibacterial properties of silver nanoparticles

In vitro antibacterial studies

The antibacterial assay was done by disc-diffusion assay method (Casida 1986). In this method, 50 μ l of silver nanoparticle prepared from leaf extract, was mixed in 1 ml of distilled water and applied to sterile paper discs of 5 mm diameter (Hi media, India). Similarly, 50 μ l water extract of *Prosopis chilensis* mixture was applied to sterile paper discs to find out the activity of the plant extract. Sterile

paper disc without any treatment was used as control. The discs were then placed on Muller Hinton agar, swabbed with different test pathogens such as *Vibrio cholera*, *V. harveyi*, and *V. parahaemolyticus* which was prepared at a concentration of 10^6 bacteria/ml in nutrient broth. These bacterial strains were isolated earlier from infected shrimps and identified. The plates were incubated at 30°C for overnight. The zones of inhibition were measured after the 24 h of incubation.

In vivo antibacterial studies

A batch of healthy adults *Penaeus monodon* (mean body weight 15.6 ± 1.5 g) were brought to the laboratory from a shrimp farm located at Parangipettai. The shrimps, after 7 days of quarantine, were transferred to plastic tanks of 50 L capacity and acclimatized for a week. The experiment was conducted for 30 days in the laboratory conditions. Physico-chemical parameters such as salinity, $\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$ and dissolved oxygen were monitored regularly for the rearing water which is maintained at optimal levels by water exchange. The selected healthy shrimps were divided into four different groups. Each contained a total of 15 shrimps.

Group I: Control (without any treatment)

Group II: Fed with silver nanoparticles alone (0.1 mg of silver nanoparticles in 10 mg of standard feed)

Group III: Added with *Vibrio* species mixture (10^6 counts/ml) and fed with silver nanoparticles (0.1 mg of silver nanoparticles in 10 mg of standard feed)

Group IV: Added with *Vibrio* species mixture (10^6 counts/ml)

The animals in group I provided with standard feed without any addition were considered as control. The animals of group II and III were applied with silver nanoparticle solution by mixing it with the standard feed (0.1 mg of silver nanoparticles in 10 mg of standard feed) and air dried. The group II was applied with silver nanoparticles alone whereas group III was applied with silver nanoparticles and *Vibrio* species mixture. The *Vibrio* species mixture was made by culturing, *V. cholerae*, *V. harveyi*, and *V. parahaemolyticus* in Zobell marine broth and cell biomass was harvested at the 18 h of incubation. Cells were separated by centrifugation at 7,500g for 10 min at 4°C and mixed in water at a concentration of 10^6 counts/ml. The animals in group IV were treated with *Vibrio* species mixture and were provided with standard feed alone. Feed was given twice daily (8 a.m. and 7 p.m.) for 2 days at a rate of 10–15% of body weight per day. The percentage of survival rate was monitored daily during days of the experiment by counting the number of the animals died. The immune parameters were assayed on the initial and after 30 days of experiment.

Haematological and immunological parameters

Haemolymph was collected from experimental shrimps aseptically from the rostral sinus using a sterile capillary tube having a diameter of 0.5 mm and pre-rinsed with anticoagulant, EDTA. The haemolymph was transferred to sterile microcentrifuge tubes containing the anticoagulant. Total haemocyte count (THC) was made using a haemocytometer and expressed as THC ml^{-1} haemolymph.

Phenoloxidase activity was measured spectrophotometrically using L-3, 4-dihydroxyphenylalanine as the substrate (Soderhall 1981). The dopachrome formed was measured at 495 nm and phenoloxidase activity was then expressed as the increase in absorbance per minute per 100 μl haemolymph. The endobiotic assay was conducted using haemolymph (50 μl) collected from different groups of experimental shrimps. The haemolymph was loaded on sterile paper discs and placed on a Muller Hinton agar plate, swabbed with bacterial strains (*Vibrio cholerae*, *V. harveyi*, and *V. parahaemolyticus*). The plates were incubated at 30°C for 18 h and diameter of the growth inhibition zones was measured.

Statistical analysis

Statistical analysis was performed using one way analysis of variance by SPSS software package (version 17.0), followed by Duncan's multiple range test (DMRT). Results are expressed as mean \pm standard deviation from six shrimps in each group.

Results

Synthesis of silver nanoparticles

The reaction mixture consisted of silver nitrate and leaf extract exhibited change of colour, as noted by visual observation. The plant extract incubated with silver nitrate at the beginning of the reaction is shown in Fig. 1a. After the incubation in dark, the colour of the reaction mixture showed gradual change into yellowish brown, with intensity increasing during the period of incubation and was finally turned to intense brown after 24 h of incubation (Fig. 1b). Control (without silver nitrate) showed no change in colour of the leaf extract. The colour intensity in terms of optical density of the reaction mixture was taken at different wavelengths ranging from 300 to 700 nm and found that the absorbance peak was at 420 nm (Fig. 2).

Characterization of silver nanoparticles

In order to confirm the nature of silver nanoparticles produced by coastal *Prosopis chilensis*, X-ray diffraction

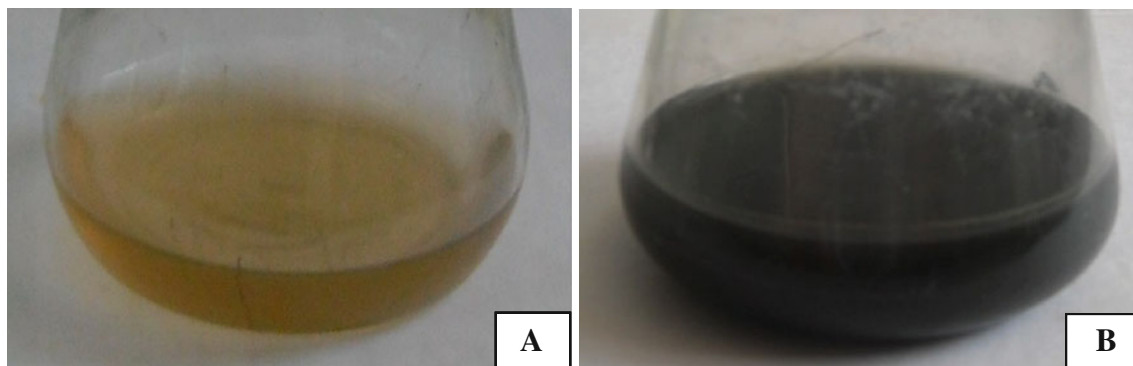


Fig. 1 Leaf extract of *Prosopis chilensis* with silver nitrate (1 mM): **a** at the beginning of the reaction and **b** after 24 h of reaction

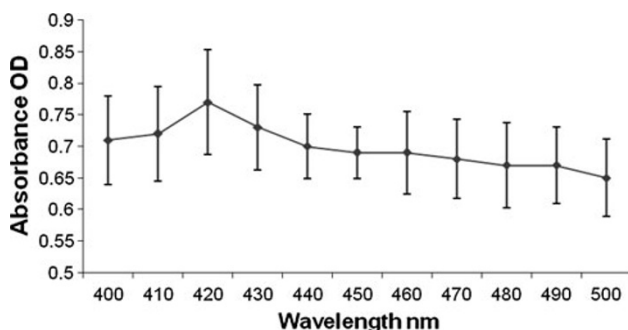


Fig. 2 Absorption spectrum for silver nanoparticles produced by coastal *Prosopis chilensis* at a range of wavelengths after 24 h of incubation (Each value is mean \pm standard deviation for three replicates)

pattern was found out as shown in Fig. 3. The XRD pattern showed intense peaks in the whole spectrum of 2θ value ranging from 20 to 80 with four intense peaks (111), (200), (220), (311) supporting strong Bragg reflections which correspond to the reflections of fcc silver. The silver nanoparticle was then analyzed for FTIR spectra. The Fig. 4 shows

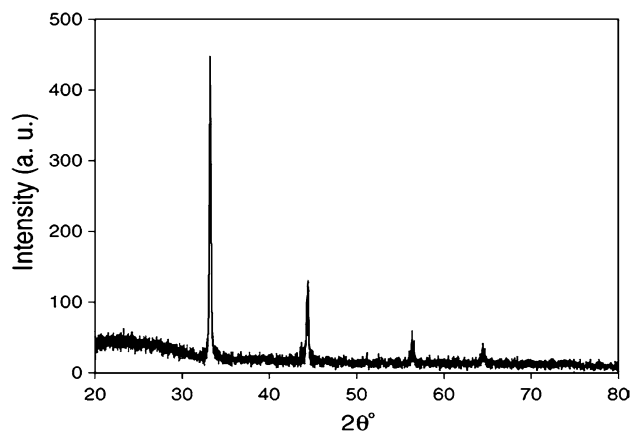


Fig. 3 XRD pattern of silver nanoparticles produced by leaf extract of coastal *Prosopis chilensis*

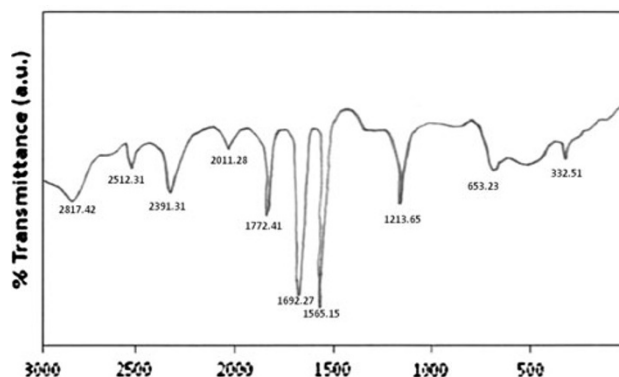


Fig. 4 FTIR spectrum of silver nanoparticles produced by leaf extract of coastal *Prosopis chilensis*

peaks at 1,565.15, 1,692.27 and 1,772.41 (C=C groups or from aromatic rings). The shape and size of silver nanoparticles obtained after 24 h of incubation was then confirmed by TEM micrograph as presented in Fig. 5. The micrograph showed nanoparticles with variable shape, most of them

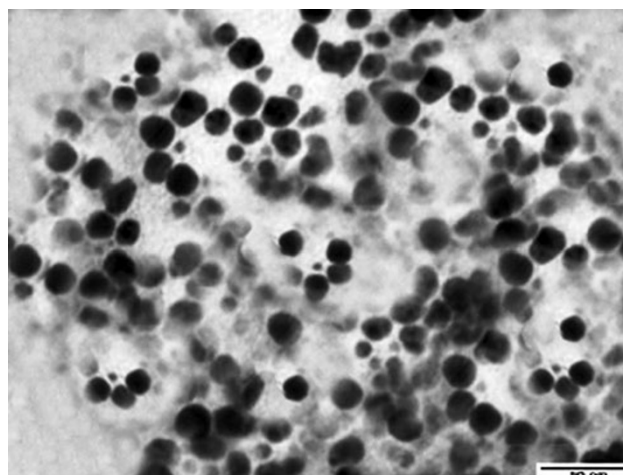


Fig. 5 TEM micrograph of silver particles synthesized by leaf extract of coastal *Prosopis chilensis* (scale bar 50 nm)

were of spherical in nature with varied size which ranged from 5 to 25 nm with an average size of 11.3 ± 2.1 nm. The particle size distribution histogram of silver nanoparticles from transmission electron microscope (TEM) analysis are given in the Fig. 6.

In vitro antibacterial studies

The discs applied with silver nanoparticle inhibited significantly all the vibrio pathogens *V. cholerae*, *V. harveyi*, and *V. parahaemolyticus* tested with inhibition zone of 22 ± 3.5 , 16 ± 2.7 and 19 ± 2.8 mm, respectively. In contrast, there was no antibacterial activity in control and only a trace of activity with inhibition zone of less than 1 mm in the case of leaf extract (Table 1). Hence there was a statistically significant variation in antibacterial activity between each groups against the three pathogens tested ($P < 0.05$).

Survival of shrimps

After 30 days of experiment, the percent survival of shrimp with different groups is shown in Fig. 7. Shrimps fed with silver nanoparticles showed higher survival rate. The silver

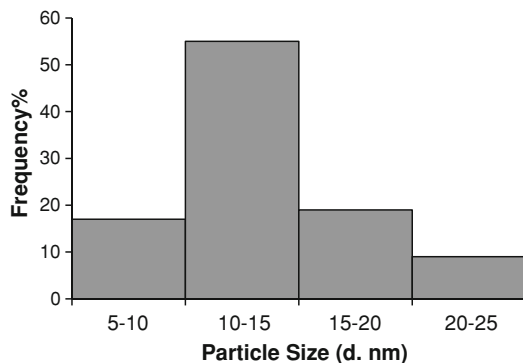


Fig. 6 Particle size distribution histogram of silver nanoparticles from transmission electron microscope (TEM) analysis

Table 1 Antibacterial activity of leaf extract of *Prosopis chilensis* and silver nanoparticles produced by the plant against vibrio pathogens on disc diffusion assay

Disc impregnated with	Inhibition zone (mm)		
	<i>V. cholerae</i>	<i>V. harveyi</i>	<i>V. parahaemolyticus</i>
Leaf extract	<1	<1	<1
Silver nanoparticles produced by leaf extract + AgNO ₃	22 ± 3.5	16 ± 2.7	19 ± 2.8
Control	0	0	0

Values mean \pm standard deviation from three replicates in each group

nanoparticle-fed shrimps when challenged against *Vibriosis* exhibited a similar survival to that of control. In contrast the vibrios alone treated group showed total mortality at the end of the culture period (Fig. 7).

Immunomodulatory effect

The haemocyte counts and phenoloxidase were observed at higher levels in the shrimps fed with nanoparticles, exhibiting 92.8 and 185.7% increase of haemocytes and enzyme, respectively in 30 days of experiment (Tables 2, 3). In this experimental shrimp, haemolymph showed high antibacterial activity of 4.3, 8.4 and 5.3 mm against *V. cholerae*, *V. harveyi* and *V. parahaemolyticus*, respectively (Table 4). There was a decline in haemocyte count by 38.2% and phenol oxidase by 18.6% and very low antivibrio activity of haemolymph in the shrimps challenged with vibrios. In contrast, the vibrios-challenged shrimps after feeding with silver nanoparticles exhibited a similar result which is on par with that of control.

Discussion

The modernization and intensification of aquaculture industry lead to outbreak of vibriosis resulting in huge loss of aquaculture industries throughout the world. The present study was designed to find the potential of silver nanoparticles, and to control vibriosis in the culture of *P. monodon*. The silver nanoparticles used for the present study were biosynthesized from *Prosopis chilensis*. Other plants such as *Azadirachta indica* (Neem) (Shankar et al. 2004), *Medicago sativa* (Alfalfa) (Gardea-Torresdey et al. 2003), *Aloe vera* (Chandran et al. 2006) and *Emblia officinalis* (amla, Indian Gooseberry) (Ankamwar et al. 2005) are already known for nanoparticle synthesis. The

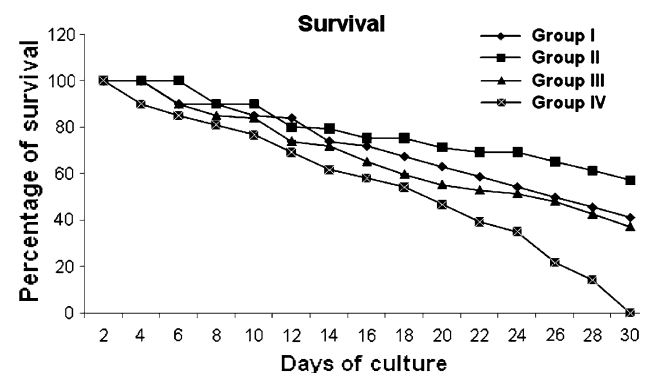


Fig. 7 Survival (%) of *Penaeus monodon* in experimental shrimps: (I) control, (II) fed with silver nanoparticles, (III) fed with silver nanoparticles and challenged with vibrios, (IV) challenged with vibrios

Table 2 Total haemocyte counts of shrimp, *Penaeus monodon* in different experimental groups

Groups	Total haemocyte counts (cells. ml ⁻¹)		
	0 day	30 days	% change
Group 1 (control)	80 ± 5.5 ^a	103 ± 6.5 ^a	28.8
Group 2 (fed with silver nanoparticles)	83 ± 5.1 ^a	160 ± 9.8 ^b	92.8
Group 3 (fed with silver nanoparticles and challenged with vibrios)	81 ± 5.3 ^a	107 ± 6.3 ^a	32.1
Group 4 (challenged with vibrios)	89 ± 3.4 ^a	55 ± 2.4 ^c	-38.2

Values mean ± standard deviation from three replicates in each group

Values not sharing a common superscript differ significantly ($P < 0.05$, DMRT) between time intervals

Table 3 Phenol oxidase activity of shrimp, *Penaeus monodon* in different experimental groups

Groups	Phenol oxidase activity (OD at 495 nm)		
	0 day	30 days	% change
Group 1 (control)	0.52 ± 0.04 ^a	0.81 ± 0.09 ^b	55.8
Group 2 (fed with silver nanoparticles)	0.49 ± 0.03 ^a	1.4 ± 0.1 ^c	185.7
Group 3 (fed with silver nanoparticles and challenged with vibrios)	0.51 ± 0.04 ^a	0.77 ± 0.06 ^b	50.9
Group 4 (challenged with vibrios)	0.43 ± 0.03 ^a	0.35 ± 0.04 ^a	-18.6

Values mean ± standard deviation from three replicates in each group

Values not sharing a common superscript differ significantly ($P < 0.05$, DMRT) between time intervals

Table 4 Antibacterial activity of haemolymph of shrimp, *Penaeus monodon*

Groups	Inhibition zone (mm)		
	<i>V. cholerae</i>	<i>V. harveyi</i>	<i>V. parahaemolyticus</i>
Group 1 (control)	Trace	2.3 ± 0.04 ^a	1.2 ± 6.5 ^a
Group 2 (silver nanoparticles alone)	4.3 ± 0.03 ^a	8.4 ± 0.03 ^b	5.3 ± 9.8 ^b
Group 3 (vibriosis + silver nanoparticles)	1.4 ± 0.04 ^b	2.3 ± 0.04 ^a	Trace
Group 4 (vibriosis)	0	0	0

Values mean ± standard deviation from three replicates in each group

Values not sharing a common superscript differ significantly at $P < 0.05$ (DMRT)

present study proved the biosynthesis of silver nanoparticles by the coastal *Prosopis chilensis* was successful. The synthesis of silver nanoparticles was noted by change in colour of leaf extract when added with AgNO₃ and the colour change was due to excitation of surface plasmon vibrations, which indicated formation of silver nanoparticles (Mulvaney 1996).

It is important to know the exact nature of the silver particles formed by measuring the XRD-spectrum of the samples. The XRD-spectrum measured in this case resulted in four intense peaks (111), (200), (220) and (311) (Fig. 3), which are in agreement to the Bragg's reflection of silver nanocrystals (Lu et al. 2003). This confirmed that the silver

nanoparticles formed by the extra cellular reduction of Ag⁺ ions by *Prosopis chilensis* are crystalline in nature.

The FTIR measurement helps in finding out the possible biomolecules, responsible for capping and efficient stabilization of the metal nanoparticles. The FTIR spectra of nanoparticles (Fig. 4) exhibited peaks at 1,565.15 cm⁻¹ (carboxylate anion stretch mode), 1,692.27 (amide I) and 1,772.41 (C=C groups or from aromatic rings) suggesting the presence of flavanones, terpenoids and other methoxy compounds with minor peaks. The results of the present study reveal that flavanones or terpenoids adsorbed on the surface of metal nanoparticle, possibly by interaction through carbonyl groups or π -electrons in the absence of

other strong ligating agents in sufficient concentration. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acids, suggested by Shankar et al. (2004) while studying the synthesis of bimetallic Au–Ag nanoparticles using *Azadirachta indica* leaf extract.

The present study attempted antibacterial activity of the silver nanoparticles and found that they inhibited the growth of all vibrio species tested (Table 1) in the in vitro studies. The in vivo experiment which was conducted on shrimp, *Penaeus monodon* for 30 days revealed that the shrimps fed with silver nanoparticles exhibited higher survival rate. These shrimps when challenged with vibrios showed a similar survival rate to that of control. In contrast, there was no survival of the shrimps challenged with vibrios at the end of the culture period (Fig. 7). Increasing *Vibrio* population in animal reduced the survival rate (Singh 1986; Hameed 1993). This reveals that the application of silver nanoparticles resulted in decreased counts of vibrios and hence the higher survival rate of the shrimps (Singh 1986; Hameed 1993).

The haemocytes have several important functions including prevention of haemolymph loss by coagulation, immobilization of pathogens by clotting, phagocytosis of foreign material, and hardening of cuticle (Soderhall and Smith 1986). Total haemocyte counts are sporadically used by shrimp disease diagnosticians (Lightner 1996). Haemocyte counts from 10^4 to 10^5 cell/mm³ was maintained in healthy *P. monodon*, but when infected, there is a marked drop in counts (Supamattaya et al. 2005). In the present study also, there was a decline in haemocyte counts of shrimp group challenged with vibrios but counts increased in the shrimps fed with silver nanoparticles (Table 2). This is because the adverse effect caused by the vibrio, which was reduced by the application of silver nanoparticles.

The most well-studied haemolymph defensive system of crustaceans is phenoloxidase activity. Phenoloxidase catalyses the oxidation of the phenols to quinones, which subsequently polymerizes into a black-pigmented polymer, called melanin. Phenoloxidase is stored in its inactive form as prophenol oxidase (pro-PO), in haemocytes of crustaceans (Albores et al. 1993). Some of the intermediates in this melanization reaction are toxic to the microbial pathogens (Soderhall and Ajaxon 1982). In the present study, a prominent elevation in phenoloxidase level was observed in haemolymphs of the shrimps fed with silver nanoparticles (Table 3).

Broad-spectrum antibacterial activity is known in haemolymph of many crustaceans including species of lobsters, shrimp, crabs, isopods and stomatopods. Factors responsible for the antibacterial activity of haemolymph are collectively called as endobiotics which reside inside haemocytes and they are released after appropriate

stimulation (Khoo et al. 1999). The present study also supported the above statement that the haemolymph showed antibacterial activity which was well pronounced when silver nanoparticle was applied (Table 4). Several mechanisms of inhibitory action of silver ions on microorganisms have been proposed. DNA loses its replication ability (Feng et al. 2000) and expression of ribosomal subunit proteins, as well as, some other cellular proteins and enzymes essential to ATP production becomes inactivated by silver ions (Yamanaka et al. 2005). It is hypothesized that silver primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain (Bragg and Rainnie 1974; McDonnell and Russell 1999). The bactericidal activity of silver nanoparticles is due to interaction between the nanoparticles and constituents of the bacterial membrane which causes structural changes and damage to membranes, finally leading to cell death (Sondi and Salopek-Sondi 2004).

Silver-based antibacterial agents receive much attention because of the low toxicity of the silver ions to human cell (Williams et al. 1989; Berger et al. 1976), as well as, it is a long-lasting biocide with high thermal stability and low volatility. Another beneficial effect of using silver is due to the fact that pathogens cannot mutate to avoid its antibacterial effect. The development of resistance to antibacterial silver would be extremely rare because an organism would have to undergo simultaneous mutations in every critical function within a single generation to escape its influence. Spontaneous mutation is rare, occurring in only 1 per 10^5 divisions, so the probability of multiple-dependent mutations occurring in the same generation of microbes is extremely small (Gibbins 2003). This problem is, however, common with antibiotics; bacteria carrying mutations causing antibiotic resistance pose a threat to human health, as they do not react to conventional treatment and may horizontally transfer this trait to other bacteria. The inhibition produced against the *vibrio* species would not cause any problem of antibiotic-resistant bacteria. Moreover, the fact that silver affects a number of cellular processes as well as the membrane integrity, while antibiotics specifically target one process. The bacteria mutate in such a way that the antibiotic is metabolized or removed from the cell. Also the specific target enzyme can be mutated, so that the sensitivity for the antibiotic decreases. In the case of silver, some of the resistance genes that have been identified seem to be involved in pumping silver out of the cell (Knetsch and Koole 2011; Cuin et al. 2007). Hence the silver nanoparticles are better antibacterial agents. In the present study, silver nanoparticles prevented vibriosis infection in the shrimp. The possible mode of action might be because silver nanoparticles inside the shrimp prevented the entry of pathogenic vibrios and hence the haemocyte counts and phenoloxidase

activity of the shrimp were stimulated (Tables 2, 3). However, the detailed mechanism should be further investigated.

The present study revealed the application of silver nanoparticles as an alternative remedy for controlling vibriosis in the culture of *P. monodon*. However, the doubt about the silver accumulation in the shrimps remains uncertain and requires detailed investigations. Although the present study used a little concentration of silver nanoparticles, it is necessary to monitor the toxic levels in the shrimp tissue as the nanomaterials become more prevalent. However, estimations of environmentally likely concentrations of silver nanoparticle are so far not available (Griffitt et al. 2009). This reveals the need for further studies focused on understanding the mechanisms of nanoparticle toxicity in aquatic organisms as warranted by Griffitt et al. (2009). Hence, it requires detailed studies on risk assessment on silver accumulation in the animals and also on the economic viability in large scale application.

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