Myrobalans Mediated Nanocolloids in Controlling Marine Pathogens

Ranjani S
B. S. Abdur Rahman Crescent Institute of Science and Technology
Pradeep Parthasarathy
B. S. Abdur Rahman Crescent Institute of Science and Technology
Rameshkumar p
Central Marine Fisheries Research Institute
Hemalatha S (hemalatha.sls@bsauniv.ac.in)
B. S. Abdur Rahman Crescent Institute of Science and Technology https://orcid.org/0000-0002-8150-7721

Research Article

Keywords: Myrobalans, silver nanocolloids, foodborne pathogens, V. harveyi, V. alginolyticus, V. parahaemolyticus, S. haemolyticus

Posted Date: August 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-772185/v1

Abstract

Aquaculture production is affected by disease outbreak, which affect the production, profitability, and sustainability of the global aquaculture industry. Antibiotics have been widely used to control various pathogens. Due to its uncontrollable and indiscriminate usage, pathogens have developed resistance towards antibiotics. This current study aims to synthesize green silver- of silver nanocolloids (MBNc) from the extract of three myrobalans. MBNc was characterized by using UV-Vis spectroscopy, DLS, Zeta potential, FE-SEM. EDX, HRTEM, and SAED techniques. The array of antimicrobial assays were performed to evaluate the efficacy of MBNc against vibriosis causing pathogens including *V. harveyi, V. alginolyticus, V. parahaemolyticus,* and foodborne pathogen *S. haemolyticus*, isolated from infected fish. Further, the presence of ESBL genes including CTX-M-15 and Amp C were analyzed in control and MBNc treated strains. From our studies, it was observed that MBNc was very effective in controlling the growth and biofilm formation in all tested marine pathogens and effectively abolish the genes encoding ESBL CTX-M-15 in antibiotic-resistant pathogens tested. Thus, MBNc can be formulated to control the growth of marine pathogens and it can be used as an alternative to antibiotics to prevent infection in cage culturing and aquafarming.

1. Introduction

Seafood is a good source of protein, vitamins, minerals, and most importantly omega 3 fatty acids which is available only through seafoods [1]. According to the statistics, 151.2 million tonnes of seafoods are consumed per year by humans. This is contributed by 11.6 million tonnes from Marine fisheries and 79.3 million tonnes from Inland fisheries. There is a huge gap in the consumption of food and production. An average person eats approximately half his or her weight of seafood each year. To meet the ever-growing need of seafood many aquaculture methods have been initiated to grow certain species in a regulated condition and the produce from these accounts for about 110.2 million tonnes per year. Seafood contribute to nearly 215 billion dollars, accounting for ~0.3% of the world economy [2].

The major concern for both aquaculture and marine fisheries is the maintenance and prevention of any infection which affects their commercial value which greatly affects the economy. Fish infections affect either directly by affecting their taste and causing associated illnesses in the host which affects the commercial value. Indirectly, infections of Coral reefs, dolphins, whales, and other ornamental fishes which impact tourism and other indirect means of economy Marine fish infections lead to reduction in potential catch due to the decreased number of live fishes available due to increased mortality slow growth, associated bad taste, lower meat quality, decreased shelf life and associated human health concerns which makes the fish products unlikely to be bought [3].

Vibrio harveyi, Vibrio alginolyticus, Vibrio parahaemolyticus cause vibriosis to all kinds of Vertebrate, especially in the vertebrate marine organisms. Vibriosis is a serious illness associated with marine fisheries and hence communicable to humans by direct consumption of uncooked fishes and shellfishes or rarely by direct exposure to seawater. The disease is accompanied by watery diarrhoea, stomach

cramps, vomiting, fevers & chills, and other problems associated with the gastrointestinal tract [4]. Due to its pathogenicity in shellfishes, oysters and other common fishes infected organisms are undesirable for purchase and causing commercial loss. In human consumption or exposure to Vibrio, resulted in severe digestive problems associated with water loss. Various exotoxins and virulence factors are associated with *Vibrio* infection in fish as well as in human that include proteases, hemolysins, phospholipases, cytotoxins, etc [5].

*V. harvey*i is a free-swimming marine aquatic bacterium which is responsible for major infections in marine vertebrates and invertebrates. *V. harveyi* is one of the causative agents of seafood spoilage. It is reported to infect Corals, oysters, prawns, penaeid shrimp, sandbar shark, lemon sharks, Jack crevalle, Sea cucumber, and variety of other fish species. Infections of *V. harveyi* associated with marine vertebrates are Deep dermal lesions in Jack crevalle (*Caranx hippos*), eye lesions, and gastro-enteritis in a variety of fish species, infectious necrotizing enteritis in Summer flounder, vasculitis in lemon sharks and Skin ulcer in Sandbar shark. Infections of *V. harveyi* associated with marine invertebrates are White spot on the foot in Japanese abalone, luminous vibriosis and bolitas nigricans in penaeid shrimps, skin ulceration in sea cucumber (*Holothuria scabra*). *V. harveyi* is associated with wound infection and necrotizing infection in humans [6].

V. alginolyticus, is a highly dominated species of *Vibrio* community, distributed throughout the marine environment. It is a Gram-negative bacterium which grows as a parasite attached to the marine fishes and human beings. *V. alginolyticus* is reported to infect *Penaeus vannamei Boone, Epinephelus coioidesi, Crassostrea rhizophorae, Pseudo sciaenacrocea,* and *Sparus latus* [7]. It is reported to cause severe infection in human beings and animals. Infection includes osteomyelitis, intracranial infection, otitis, ocular infections, and peritonitis. Sea foodborne infections include soft-tissue infections followed with necrotizing, necrotizing fasciitis, accompanied with septic shock and multiple organ dysfunction, resulting in high morbidity and mortality. The number of infections associated with *V. alginolyticus* has hiked up during the past few decades, resulting in public threats through food safety and security [8].

V. parahaemolyticus is an enteropathogen, survive both in Marine and freshwater sources. *V. parahaemolyticus* causes infections in crabs, shrimp, tail, and fin rot disease in ornamental fishes. *V. parahaemolyticus* is a major causative agent for an ear infection, wound infection, gastroenteritis, and septicemia. The infection of *V. parahaemolyticus* is caused because of the mishandling of seafoods during processing and storage, eating uncooked seafoods [9].

S. haemolyticus is not a normal microflora of marine fishes. *Staphylococci* are commensals present in the skin of humans and animals. The isolation of *Staphylococci sp*. indicates the improper packing of fish foods and rarely associated with fish diseases. *Staphylococci sp* is responsible for foodborne diseases and food poisoning because of the consumption of raw or uncooked food [10].

All the Vibriosis causing pathogens *V. harveyi, V. alginolyticus, V. parahaemolyticus,* and foodborne pathogen *S. haemolyticus*, adversely affect the marine vertebrates and invertebrates, which ultimately leads to economic loss to Aquaculture industries. Improper processing, storage of sea foods and

uncooked seafoods lead to severe life-threatening diseases to human beings. Besides, these pathogens *Vibrio harveyi, V. alginolyticus, V. parahaemolyticus*, and *S. haemolyticus* are resistant to antibiotics. During the past few decades, excessive use of antibiotics in the field of aquaculture, agriculture, and in human medicine, these organisms have developed resistance to many antibiotics. Development of multidrug resistance is one of the major impacts developed recently making the antibiotics ineffective [11]. Thus, this is the need of the hour to find the best alternative to protect the whole society through an environmentally friendly nano solution.

Antimicrobial property of Silver Nanoparticles, nanocolloids and nanosuspensions have already been well established and numerous findings have proven for its efficacy in treating many multidrug-resistant pathogens such as *Pseudomonas aeruginosa, Streptococcus sp.* Methicillin resistant *Staphylococcus aureus*, *E.coli*, and other extended-spectrum - β lactam producing bacteria (ESBL). Several mechanisms have been identified on how Silver nanoparticles interact and eliminate the bacterial infection by interfering certain biochemical pathways [12].

In our study, we focus on the green nano colloidal system, which uses a combination of herbal extract for the synthesis of green nanocolloid. This green nanocolloids has high antimicrobial efficacy against many infections due to its combined effect of biological extract enriched with polymeric macromolecules and other invaluable polymeric phytocompounds and silver. Many plant species have been used from ancient days as an effective antimicrobials. Plants and its products enhance the defence mechanism through enriched secondary metabolites and other biologically important macromolecules. Thus, the biological synthesis of green silver- nanocolloids using myrobalans extract has multiple advantages to all living beings, without having any deleterious effect to the environment.

Triphala, a combination of three myrobalans of Indian origin (Equal amount of *Emblica officinalis, Terminalia belerica, and Terminalia chebula*) is a traditional Ayurvedic herbal medicine which has been utilized as antioxidant, anti-inflammatory, antimicrobial medicine. The increased content of flavonoids, phenols, polyphenols, proteins, carbohydrates and other phytochemicals provide the increased reducing capability and it could be incorporated for biosynthesis of green nanocolloids [13]. Although antimicrobial property of Triphala has been well elucidated and studied in detail in ayurvedic medicine, little information is available pertaining to the combinatorial effect of green Silver nanocolloid system and Triphala extract. This research paper is focussed on combinatorial effect of biologically synthesised green silver Triphala nanocolloid system in controlling marine pathogens, which are adversely affecting the aquaculture industry and human being through its fish borne pathogens.

2. Materials And Methods

Pathogenic strains of *V. harveyi, V. alginolyticus, V. parahaemolyticus* and 2 strains of *S. haemolyticus* were obtained from Central Marine and Fisheries Research Institute, Mandapam.

2.1 Antibiogram assay

Antibiotic susceptibility of the pathogenic strains was checked by testing the strains of *V. harveyi, V. alginolyticus, V. parahaemolyticus* and 2 strains of *S. haemolyticus* using antibiotic discs such as Penicillin 10µg, Ampicillin 2µg, Enrofloxacin 5µg, Gentamycin 30µg, Ceftriaxone 30µg, Streptomycin 10µg, Cefoperazone sulbactam 75µg (HiMedia, India)). The cultures were grown as per McFarland standard and inoculated on Muller Hinton agar plates. All the antibiotic discs were placed in the proper orientation and incubated at 37^{o}C overnight. The zone of inhibition was measured and recorded. The strains which do not show any zone of inhibition is recorded as resistant and strains with a clear zone of inhibition was categorized as susceptible [14].

2.2 Preparation of Triphala extract of and qualitative phytocompounds analysis

The extracts of Triphala (Equal amount of three Myrobalans *Emblica officinalis, Terminalia belerica, Terminalia chebula*) was prepared by mixing 10gm of myrobalans powder with 100 ml of autoclaved water and kept at 37ºC shaker incubator overnight. The solution was then boiled in the hot plate for 20-30 min. The solution is then cooled and allowed to settle down followed by filtration using Whatmann No 1 filter paper and stored at 4ºC for further use [15-18]. The myrobalans extract was subjected to qualitative phytochemical analysis [19] and quantitative carbohydrate analysis using Anthrone method [20].

2.3 Myrobalans mediated nanocolloids synthesis and physicochemical characterization of MBNC

Myrobalans mediated synthesis of green nanocolloids using Myrobalan extract (MBNc) was carried out by mixing 100 ml of enriched aqueous extract with 500ml of 1mM AgNO3 (1:5) followed by incubation in dark at the static condition at room temperature. The noticeable colour change was monitored periodically for the synthesis of MBNc. UV-Vis spectroscopy was used to confirm the MBNc synthesis. The spectral scan was carried out between 300 to 700nm. The maximum SPR peak confirmed the synthesis of MBNc. The synthesized silver nanocolloid was purified by washing the pellet with autoclaved water, followed by centrifugation. Pellet was then resuspended in 10% Dimethyl Sulfoxide (DMSO) and sonicated for 5 minutes in ice for uniform distribution and utilized for further characterization [21-23].

The size of MBNc was determined using Zeta sizer. The size of the particle was measured based on the light scattering measurement carried out during the dynamic motion of the nanoparticles present in MBNc for 1 minute at 25^{o}C. The graph displays the hydrodynamic diameter and PDI of the nanocolloidal particles. Zeta potential values are measured using the same instrument integrated with Zeta potential analyzer. The shape, size, and geometry of the nanocolloid were determined using FESEM. EDAX integrated FESEM depicts the elemental composition of nanocolloidal particles [24-26]. HRTEM measurement was carried out by placing the drop of MBNc on copper grids and incubated under desiccation. The grid was loaded on the sample holder and HRTEM was taken using HRTEM: Jeol/JEM 2100 of 200kV under LaB6 Electron gun with 0.23nm resolution and lattice resolution 0.14 nm integrated with SAED [24-26].

2.4 Antimicrobial property of biologically synthesized green nanocolloids (MBNc) against marine pathogens

The antimicrobial potential of MBNc was validated through four different assays namely, bacteriostatic assay (Minimum inhibitory concentration), time kill curve validating through different concentrations of MBNc, and Bactericidal assay (Minimum bactericidal concentration) and antibiofilm assay.

The fresh cultures of *V. harveyi, V. alginolyticus, V. parahaemolyticus,* and 2 different strains of *S. haemolyticus* were grown in LB broth and all the experiments were carried out at 0.5 McFarland standard.

2.4.1 Agar well diffusion Method

The preliminary antimicrobial activity of extract of myrobalans and MBNc was analyzed by the agar well diffusion method. The fresh culture was swabbed evenly on LB agar plates and allowed to dry. Then the wells were punctured using sterile 6mm tips. Each well was loaded with positive control Ampicillin, myrobalan extract, and 25µl and 50µl of MBNc. All the plates were incubated at 37^{o} for overnight. The zone of inhibition was measured and recorded [21].

2.4.2 Bacteriostatic assay and Time kill curve

The bacteriostatic assay was performed to calculate the minimum concentration of MBNc required to inhibit the growth of the test organisms. The bacteriostatic assay was performed using the broth dilution method with some modification. The assay was performed in 96 well microtiter plates. 100µl LB broth filled in each well of the 96 well plates. MBNc (1mg/ml) was serially diluted in all wells allotted for treatment (100µg to 0.78µg). Ampicillin 25µg was added to corresponding wells as a positive control. The untreated wells are served as control. Microtiter plates were incubated at 37ºC. Bacteriostatic concentration was calculated by observing the visual turbidity of the growth medium. After 24 hours of incubation, the wells with invisible growth was chosen for minimum inhibitory concentration. To observe the growth rate of organisms at different concentrations, OD at 600nm was taken at various time intervals from 0h, 12 h, 18h, 24h, 30h, 36h, and 48h. By plotting the graph, the growth rate of the organism under MBNc treatment was calculated and compared to control and Ampicillin treatment [27].

2.4.3 Bactericidal assay

The bactericidal assay was performed to arrive at the concentration that completely kills test organisms. The bactericidal assay was continued with the same microtiter plate used to calculate the bacteriostatic concentration. 2µl of cultures from each well with invisible growth, even after 24 hours of MBNc treatment were chosen for each strain and inoculated in LB agar plates. The plates were then incubated at 37ºC for overnight. The minimal concentration of MBNc which completely kills organisms were recorded as bactericidal concentration [24- 28].

2.4.4 Biofilm inhibition assay

Biofilm assay was carried out in microtiter plates, similarly as a bacteriostatic assay. After 48 hours of incubation bacterial growth was stopped by discarding the used broth. The plates were washed thoroughly with distilled water to remove the planktonic bacteria. The plates were dried completely and 200µl of 0.1% crystal violet was added to all wells and placed in the rocker for 15 minutes. After complete staining of biofilms, the crystal violet was discarded and washed twice with distilled water. The plates are then dried completely. Followed by the addition of 200µl of 30% Glacial acetic acid to dissolve the crystal violet stain over the biofilm and absorbance was measured at 595nm using a Multimode plate reader (EnSpire Multimode Plate Reader from Perkin Elmer, USA). The absorbance value directly correlates with the amount of biofilm formed in MBNc treated and untreated control. The assay was performed in triplicates to carry out statistical analysis by using a t-test to identify the significant difference among Ampicillin and MBNc treated strains [29- 30].

2.4.5 Analysis of CTX-M-15 and AmpC gene upon MBNc treatment

Genomic DNA was isolated from control, MBNc and Ampicillin treated strains of *V. harveyi, V. alginolyticus, V. parahaemolyticus,* and 2 strains of *S. haemolyticus*. Template DNA was amplified with a specific set of primers for CTX-M-15 and AmpC gene as per the reaction condition adopted [31]. The PCR product was run using agarose gel electrophoresis to validate the presence of genes upon treatment with MBNc and Ampicillin when compared with the control [32].

3. Results And Discussion

3.1 Antibiotic Susceptibility of Marine Pathogens

Antibiotic susceptibility of the pathogenic strains *V. harveyi, V. alginolyticus, V. parahaemolyticus* and *S. haemolyticus* were tested using antibiotic discs such as Penicillin 10µg, Ampicillin 2µg, Enrofloxacin 5µg, Gentamycin 30µg, Ceftriaxone 30µg, Streptomycin 10µg, and Cefoperazone sulbactam 75µg. From the result, it was identified that MP3, MP4, MP5 tested organisms showed resistance to Penicillin and Ampicillin and were susceptible to all other antibiotics tested (Table 1) wherease *V. harveyi* (MP1) strain was resisitant to Penicillin 10µg, Ampicillin 2µg, Enrofloxacin 5µg, and Ceftriaxone 30µg.

3.2 Phytochemical analysis of biological extract of Myrobalans

Phytocompounds enriched extracts of Triphala myrobalans was prepared and concentrated. Phytochemical analysis of biological aqueous extract of Triphala myrobalans revealed the presence of carbohydrates, phenols, saponins, terpenoids, flavonoids, alkaloids, terpenoids, anthocyanin, steroids, and carotenoids (Table 2). Medicinal plants produce phenols, flavonoids, terpenoids, and tannins that contribute to their medicinal property. Flavonoids in combination with phenols, polyphenols, saponins, and other tannins exhibit potential antibacterial, anti-tumor, anti-viral properties [33]. Antioxidant and reducing properties of these phytoconstituents help in the reduction of silver nitrate into nanocolloidal silver. The quantitative estimation of carbohydrates confirmed the presence of total carbohydrate (90mg/ml) of concentrated triphala extract. This confirmed the presence of carbohydrates which may

include the presence of all polysaccharides. The cellulose is one of the carbohydrate which can be extracted easily through water and accounts for major proportion in the Triphala extract. Previous studies have reported the presence of cellulose as a major biochemical constituent in triphala extract [34]

3.3 Biogenesis of Myrobalans mediated green nano colloids (MBNc) and physicochemical characterization

Myrobalans mediated green nano colloid (MBNc) was prepared by mixing the concentrated extract with 1mM Silver Nitrate and was kept in dark. Initial colour change was observed from pale brown that gradually changed into brownish green to dark brown nanocolloidal particles (Fig. 1a). This colour change is the preliminary signature which indicates the synthesis of biomolecules mediated nanoparticle synthesis in green colloidal system. At macroscale, the electrons are loosely packed, at nanoscale the electrons are lightly packed with restricted movement, so the intensity of scattering light varies when compared to macroscale and nanoscale. Hence, the nanoscaled particles look dark brown colour and as an indicator for the successful synthesis of nanocolloids [35]. Phytoconstituents such as starch, cellulose, phenols, poly phenols, flavonoids etc. act as reducing and capping agents and aid in the formation of nanoparticles containing colloidal solution. Further, the synthesis of myrobalans mediated green nanocolloids (MBNc) was further confirmed by physicochemical characterization.

UV-Visible spectrometer scanning was performed between 300-800nm. Silver nanoparticles have strong absorption and scattering of light. When the nanoparticles are exposed to particular wavelength, because of conduction of electrons, they undergo oscillation. The oscillation created by nanoparticle will be much stronger than non-plasmonic particles [35]. The oscillation developed by the nanoparticles, will develop strong peak, which is called as SPR peak. The SPR peak of MBNc was observed around 430nm which corresponds to nanoparticles which are spherical in shape (Fig. 1b). Myrobalans extract is reported to have phytocompounds, polymers of carbohydrates, polysaccharides, polyphenols, enzymes, coenzymes and other proteins which act as a bioreductant in reducing the silver ions [24- 27].

The size of MBNc was found as 407.1nm in range, based on Brownian movement of the nanoparticles dispersed in the colloidal solution with the poly dispersive index of 0.299 with intercept of 0.785 (Fig. 1c). The size of MBNc were measured using DLS which was based on random dynamic motion of the particles. PDI value of 0.299 is a highly acceptable value, described as homogenous. The Zeta potential value of MBNc was found as -13.5 with the conductivity of 0.497ms/cm, with the viscosity of 0.8872cP (Fig. 1d). The negative value implies that the negative surface charge of -13.9 will create a repulsive force between the particles and prevent agglomeration in the dispersed medium. The size, charge, and aggregation nature of the MBNc mainly depends on natural polymers and phytocompounds present in myrobalans extract. These phytocompounds and biological macromolecules uniformly laid over the surface of nanoparticles protect as a cap, to prevent the agglomeration. Thus, these biomolecules work synergistically along with silver in imparting effective antibacterial activity [21- 23].

FESEM images revealed that the nanocolloids were embedded with spherical shaped nanoparticles at 1µm scale (Fig. 2a). The mixture of three myrobalans may produce these spherical shaped nanoparticles,

which may have its unique role in targeting the bacterial cells through its shapes that helps in penetrating through the bacterial cell wall. EDAX analysis revealed the presence of silver at 3Kev, which confirmed the presence of silver (Fig. 2b). The histogram of EDAX analysis depicts the percentage weight and percentage atom of elements present in MBNc (Fig. 2c). The presence of other element such as cl, which works synergistically with silver in enhancing its antimicrobial activity [36, 37].

HRTEM analysis showed the distribution, average size and shape of the MBNc. HRTEM images of MBNc revealed its polydispersed and spherical (Fig. 3a) nature. The average size of MBNc was found as 23.63nm. SAED patterns are signature for the crystals. Shows the spots which indicate polycrystalline (Fig. 3b) nature of MBNc [29].

3. 4. Antibacterial activity of MBNc

3.4.1 Detection of antibacterial activity of MBNc through agar well diffusion assay

The preliminary antibacterial effect of MBNc was evaluated against *V. harveyi* (MP1), *V. parahaemolyticus* (MP2), *V. alginolyticus* (MP3) and *S. haemolyticus* (MP4 and MP5). At 50µg concentration of MBNc maximum zone of inhibition was observed as 26mm, 25mm, 26mm, 20mm for *V. harveyi* (MP1)*, V. parahaemolyticus* (MP2), *V. alginolyticus* (MP3) and *S. haemolyticus* (MP4 and MP5) respectively (Table 3). MBNc when loaded into the wells, diffuses into the maximum area. One of the property of nanoparticles is large surface area where nanoparticles encounter microorganism in maximum area, which inhibits and kills the organism through several mechanisms [38]. The synergistic effect of phytocompounds and silver enhances the antibacterial potential of MBNc.

3.4.2 Detecting the Bacteriostatic and Bactericidal concentration of MBNc

MBNc was validated to detect the concentration at which it completely inhibits the visible growth of organism. The minimum inhibitory concentration for *V. harveyi* (MP1), *V. parahaemolyticus* (MP2), *V. alginolyticus* (MP3) was found as 6.25 µg, 1.56 µg, 3.125 µg respectively, and for *S. haemolyticus* (MP4 and MP5) bacteriostatic concentration was found as 12.5µg. From this result it was observed that each strain responds differently to MBNc. The cell wall of Gram-negative *Vibrio sp* requires maximum 6.125µg of MBNc when compared to Gram-positive *Staphylococcus haemolyticus*, which requires 12.5µg of MBNc (Table 4). Because the cell wall of Gram-positive strains will be thicker than Gram-negative strains. The mode of action of MBNc will require higher concentration to penetrate the cell wall. The bactericidal concentration of MBNc was found as 12.5 µg, 3.125 µg, 6.25 µg for *V. harveyi* (MP1), *V. parahaemolyticus* (MP2), *V. alginolyticus* (MP3) respicteively and 12.5 µg for other 2 strains of *Staphylococcus haemolyticus*. From the result it was found that MBNc has potent bacteriostatic and bactericidal property against antibiotic-resistant, Gram positive and Gram-negative organisms, even up to the concentration of 12.5µg/ml (Table 4). Because nanocolloids target the bacterial cell through several mechanisms, the first target is the cell membrane. When the nanoparticles interact with the bacterial cell it creates pits on the surface of cell membrane and disrupt membrane permeability. Then the silver ions capped with biomolecules enter the cell through porins, which interfere with the normal functions of the plasma

membrane [39]. Previous reports are supporting to the functioning of nanoparticles in such a way that, imbalance of pH gradient will be created which induces the leakage of H^+ ions in *Vibrio Sp* [40]. MBNc may interact with thiol group of many functional proteins and enzymes, which may ultimately inhibit the respiratory chain and block the synthesis of ATP. There is an evidence that silver nanoparticles intercalate with phosphorus and sulphur group of DNA and inhibit the replication of the cell. Further the interaction of Ag ions with signalling pathway may alter the tyrosine phosphorylation, which leads to the adverse effect on mitosis, meiosis and finally leads to cell death. Silver ions also generate more and more ROS inside the cell, which is very toxic and leads to cell death [41]. Our results suggested that the physical property such as size, shape, charge and chemical properties such as interaction within the bacterial cell of MBNc played a crucial role to exert its action as a potent bacteriostatic and bactericidal agent.

3.4.3. MBNc modulates growth kinetics in marine pathogens

The dose dependent activity of MBNc was observed in all the test strains *V. harveyi* (MP1), *V. parahaemolyticus* (MP2), *V. alginolyticus* (MP3) and *S. haemolyticus* (MP4 and MP5) after 12 hours of treatment (Fig. 4a). To validate the effect of MBNc on growth, different concentrations are used and growth rate was measured at different time interval. And with different concentrations of MBNc (1.5625µg, 3.125µg, 6.25µg and 12.5µg). At the concentration of 1.5625µg the growth rate was decreased by 72%, 81%, 67.5%, 76%, and 61.5% in MP1 to MP5 strains, respectively. At the concentration of 3.125µg the growth rate was decreased by 73.8%, 89.8%, 88.1%, 78.9%, and 63.3% in MP1 to MP5 strains, respectively. At the concentration of 6.25µg the growth rate was decreased by 90.4%, 93.9%, 93.9%, 86.3%, and 80.8% in MP1 to MP5 strains, respectively. At the concentration of 12.5µg the growth rate was decreased by 94.88%, 94.69%, 94.3%, 99.0%, and 93.7% in MP1 to MP5 strains, respectively.

At 24 hours the growth rate was further decreased. At the concentration of 1.5625µg the growth rate was decreased by 80.6%, 86.6%, 40.9%, 40.3%, and 68.5% in MP1 to MP5 strains, respectively. At the concentration of 3.125µg the growth rate was decreased by 82%, 89%, 64.4%, 66.5%, and 83.7% in MP1 to MP5 strains, respectively. At the concentration of 6.25µg the growth rate was decreased by 90%, 90.8%, 91.0%, 82.2%, and 87% in MP1 to MP5 strains, respectively. At the concentration of 12.5µg the growth rate was decreased by 99%, 94%, 93%, 98%, and 98% in MP1 to MP5 strains, respectively. At the concentration of 12.5µg the growth rate of all the marine pathogens *V. harveyi* (MP1), *V. parahaemolyticus* (MP2), *V. alginolyticus* (MP3) and *S. haemolyticus* (MP4 and MP5) were effectively inhibited even after 24hours of incubation without any further treatment (Fig. 4b). This results suggested that MBNc could be used as potent alternate to control the growth of marine pathogens in marine fishes growing in caged farming.

3.4.4 Effect of MBNc on biofilm formation

Biofilm produced by the bacteria is a virulence mechanism which in turn become more antibiotic resistant strain. The hydrophobic nature of bacterial cell communicates and interact by means of signalling molecules to develop biofilms on the surface. In general, antibiofilm agents will disrupt the

communication through cell membrane or will quench the signals from bacterial cells that can inhibit the biofilm formation. To check this hypothesis, MBNc was validated to check the anti-biofilm action on the following marine pathogens *V. harveyi* (MP1), *V. parahaemolyticus* (MP2), *V. alginolyticus* (MP3) and *S. haemolyticus* (MP4 and MP5). From the antibiofilm assay it was found that MBNc inhibited the biofilm formation in a dose dependent manner of four different concentrations 1.5625µg, 3.125µg, 6.25µg and 12.5µg (Fig. 5a). At the concentration of 1.5625µg the biofilm formation was inhibited up to 82%, 82%, 54%, 77%, and 78.9% for MP1 to MP5, respectively. At the concentration of 3.125µg, the biofilm formation was inhibited up to 90%, 85%, 86.5%, 90.9%, and 83.6% for MP1 to MP5, respectively. At the concentration of 6.25µg the biofilm formation was inhibited up to 92%, 96.8%, 95.6%, 92%, and 88.9% for MP1 to MP5, respectively. At the concentration of 12.5µg the biofilm formation was inhibited maximum up to 97.9%, 99.7%, 97.8%, 94.9%, and 90.8% for MP1 to MP5, respectively.

As per the bacteriostatic and bactericidal assay, the MBNc was effective at the concentration of 12.5µg, supporting to these findings the MBNc also showed the maximum biofilm inhibition potential of 99%. It was reported that smaller the size of nanoparticle greater the antimicrobial effect, according to HRTEM the average size of particles was found around 23.63nm. Hence, MBNc easily penetrate the cell membrane and disrupt the surface of bacterial cell. The quorum sensors are the molecules secreted out for cell to cell communication. The secretion of these signalling compounds could have been inhibited by the nanocolloids, through inhibiting the synthesis of signalling molecules, degrading, or inactivating the signalling enzymes, analogs, disrupting the signalling cascade. MBNc can effectively disrupt the cell membrane, targets the central dogma of cell by disturbing whole mechanism of replication, transcription, and translation, sequentially all the signalling cascade will be disturbed [42]. There were previous reports supporting that nanoparticles controlling the biofilm formation in organism such as *S. dysentriae, V. parahaemolyticus and S. infestis* through interfering with cell to cell adhesion [43]. Our results suggest that MBNc can be effective anti-biofilm agent, even at lower concentration, which could be used as an effective alternative to antibiotics to control the infection caused by marine pathogen and marine associated food borne pathogens. Thus, this can be effectively used to control the usage of antibiotics in marine cage culturing and in aqua culturing industries.

3.4.5 Effect of MBNc on antibiotic resistant genes

The presence of CTX-M15 and AmpC genes was checked upon treatment with MBNc and Ampicillin. From the result it was observed that AmpC gene was absent in all the tested marine pathogens. However, CTX-M-15 was detected only in *V. alginolyticus* (MP3). Absence of CTX-M-15 gene in *V. harveyi* (MP1) *and V. parahaem*olyticus (MP2) and *Staphylococcus haemolyticus* (MP4 and MP5) suggested that virulence and antibiotic resistance were governed by some other ESBL genes present in these strains [44]. From the gel documentation, it was found that in control (without treatment), presence of CTX-M-15 gene was observed in *V. alginolyticus* (MP3). Upon treatment with ampicillin, CTX-M-15 gene was present, but upon treatment with 5µg and 10µg of MBNc, the CTX-M-15 gene was abolished completely. This demonstrates that MBNc may effectively target the CTX-M-15 gene at the transcription

and translational level and prevent the expression of CTX-M-15 gene (Fig. 5b). These results suggest that MBNc can effectively target on all strains with and without the expression of CTX-M-15 gene.

4. Conclusion

In this study, we have focused on myrobalans mediated synthesis of green nanocolloids using three myrobalans *Emblica officinalis, Terminalia belerica, Terminalia chebula,* commonly called as triphala, which is widely used in ayurvedic medicine which is of great medicinal value. MBNc showed effective antibacterial and antibiofilm activity on both Gram-positive and Gram-negative organisms, even at low concentrations. Further, MBNc suppressed the expression of the CTX-M-15 gene in *V. harveyi and V. alginolyticus*. This phytocompounds enriched biological extract of three myrobalans, mediated nanocolloids could be the potent alternative to prevent the indiscriminate usage of antibiotics in aquaculture and fish farming. The usage of MBNc also prevents the spread of the antibiotic resistant organisms to humans who are consuming the Marine foods. MBNc was formulated using traditional ayurvedic medicine, which do not cause any adverse effects when it is being used at moderate level. Further development and validation of MBNc through various formulations as nanospary, nanovaccine, Nano feed additive to prevent the pathogenic infection in aqua culturing of many economically and commercially important species such as penaeid shrimp, gilthead sea bream, European Seabass, tiger puffer, groupers, Asian seabass and Cobia.

Declarations

Acknowledgement

Funding

Authors are thankful to B.S. Abdur Rahman Institute of Science & Technology, Chennai for providing research facilities in school of life sciences. The authors also gratefully acknowledge the Ministry of Science and Technology, Department of Science and Technology (KIRAN Division) (GoI), New Delhi. (Ref No. DST/WOS-B/2018/1583-HFN (G)) and ASEAN University network (AUN)/Southeast Asia Engineering Education Development Network (SEED)/Japan International Cooperation Agency (JICA) SPRAC (SN042/MI.KU/2020).

Conflicts of interest/Competing interests

The authors declare that there is no conflict of interest

Availability of data and material

Data will be available on request

Code availability

Not Applicable

Ethics approval

Not Applicable

Consent to participate

Not Applicable

Consent for publication

All authors read and approved the manuscript for publication

Authors' contributions

SH conceived and designed research. SR, PP conducted experiments. PR contributed Pathogenic strains. SH and PR analyzed data. All authors wrote the manuscript. All authors read and approved the manuscript.

References

1. Hosomi R, Yoshida M, Fukunaga K (2012) Seafood Consumption and Components for Health. Global Journal of Health Science. doi: 10.5539/gjhs.v4n3p72
2. The state of world fisheries and aquaculture 2018.
3. Lafferty K, Harvell C, Conrad J et al. (2015) Infectious Diseases Affect Marine Fisheries and Aquaculture Economics. Annual Review of Marine Science 7:471-496. doi: 10.1146/annurev-marine-010814-015646
4. Newton A, Kendall M, Vugia D et al. (2012) Increasing Rates of Vibriosis in the United States, 1996–2010: Review of Surveillance Data From 2 Systems. Clinical Infectious Diseases 54:S391-S395. doi: 10.1093/cid/cis243
5. Osunla C, Okoh A (2017) Vibrio Pathogens: A Public Health Concern in Rural Water Resources in Sub-Saharan Africa. International Journal of Environmental Research and Public Health 14:1188. doi: 10.3390/ijerph14101188
6. Austin B, Zhang X (2006) Vibrio harveyi: a significant pathogen of marine vertebrates and invertebrates. Letters in Applied Microbiology 43:119-124. doi: 10.1111/j.1472-765x.2006.01989.x
7. Liu C, Cheng W, Hsu J, Chen J (2004) Vibrio alginolyticus infection in the white shrimp Litopenaeus vannamei confirmed by polymerase chain reaction and 16S rDNA sequencing. Diseases of Aquatic Organisms 61:169-174. doi: 10.3354/dao061169
8. Lee D, Moon S, Lee S et al. (2008) Septic Shock due to Vibrio alginolyticus in a Cirrhotic Patient: The First Case in Korea. Yonsei Medical Journal 49:329. doi: 10.3349/ymj.2008.49.2.329

9. Parthasarathy S, Das S, Kumar A (2016) Occurrence of pathogenic Vibrio parahaemolyticus in crustacean shellfishes in coastal parts of Eastern India. Veterinary World 9:330-336. doi: 10.14202/vetworld.2016.330-336
10. Czekaj T, Ciszewski M, Szewczyk E (2015) Staphylococcus haemolyticus – an emerging threat in the twilight of the antibiotics age. Microbiology 161:2061-2068. doi: 10.1099/mic.0.000178
11. Prestinaci F, Pezzotti P, Pantosti A (2015) Antimicrobial resistance: a global multifaceted phenomenon. Pathogens and Global Health 109:309-318. doi: 10.1179/2047773215y.0000000030
12. Barros C, Fulaz S, Stanisic D, Tasic L (2018) Biogenic Nanosilver against Multidrug-Resistant Bacteria (MDRB). Antibiotics 7:69. doi: 10.3390/antibiotics7030069
13. Peterson C, Denniston K, Chopra D (2017) Therapeutic Uses of Triphala in Ayurvedic Medicine. The Journal of Alternative and Complementary Medicine 23:607-614. doi: 10.1089/acm.2017.0083
14. Hemamalini V, Kavitha V, Ramachandran S (2015) In vitro antibiogram pattern of Staphylococcus aureus isolated from wound infection and molecular analysis of mecA gene and restriction sites in methicillin resistant Staphylococcus aureus. Journal of Advanced Pharmaceutical Technology and Research 6:170. doi: 10.4103/2231-4040.165019
15. Ranjani S, Shariq Ahmed M, Ruckmani K, Hemalatha S (2019) Green Nanocolloids Control Multi Drug Resistant Pathogenic Bacteria. Journal of Cluster Science 31:861-866. doi: 10.1007/s10876-019-01694-6
16. Ranjani, S. Tamanna, K. and Hemalatha, S. (2020b) Triphala green nano colloids: synthesis, characterization and screening biomarkers. Appl Nanosci. 10:1269–1279. https://doi.org/10.1007/s13204-019-01208-w
17. Ranjani, S. Faridha Begum, I. Tasneem, I.K. Senthil Kumar, N. and Hemalatha, S. (2020f) S. Silver decorated green nanocolloids as potent antibacterial and antibiofilm agent against antibiotic resistant organisms isolated from tannery effluent. Inorg. Nano-Met. Chem. https://doi.org/10.1080/24701556.2020.1811326.
18. Ranjani, S. Abdul Matheen, Antony Jenish, A. and Hemalatha, S. (2021) Nanotechnology derived natural Poly bio-silver nanoparticles as a potential alternate biomaterial to protect against human pathogens, Materials Letters. 130555, https://doi.org/10.1016/j.matlet.2021.130555.
19. Harborne J (1980) Phytochemical Methods. Springer Netherlands, Dordrecht
20. Yemm E, Willis A (1954) The estimation of carbohydrates in plant extracts by anthrone. Biochemical Journal 57:508-514. doi: 10.1042/bj0570508
21. Akther T, Hemalatha S (2019) Mycosilver Nanoparticles: Synthesis, Characterization and its Efficacy against Plant Pathogenic Fungi. BioNanoScience 9:296-301. doi: 10.1007/s12668-019-0607-y
22. Akther, T. Ranjani, S. and Hemalatha, S. Nanoparticles engineered from endophytic fungi (*Botryosphaeria rhodina*) against ESBL-producing pathogenic multidrug-resistant E. coli. Environ Sci Eur. 33, 83 (2021). https://doi.org/10.1186/s12302-021-00524-9
23. Akther, T. Khan, M.S. and Hemalatha, S. (2020) Biosynthesis of silver nanoparticles via fungal cell filtrate and their anti-quorum sensing against *Pseudomonas aeruginosa*. J. Environ. Chem. Eng.

https://doi.org/10.1016/j.jece.2020.104365

24. Ranjani, S. Rumelee Das, Shariq Ahmed, M. Esther Lalnunmawii, Senthilkumar Nachimuthu. Ruckmani, K. Hemalatha S (2020i). Myco-nanocolloids manipulate growth, biofilm formation and virulence genes in UTI causing *E. coli*, Inorg. Nano-Met. Chem. DOI: 10.1080/24701556.2020.1852426
25. Ranjani, S. Faridha Begum, I. Santhoshini, J. Senthil Kumar, N. Ruckmani, K. and Hemalatha, S. (2020j). *Mimosa pudica* floral nanoparticles: a potent antibiotic resistance breaker, Inorg. Nano-Met. Chem. DOI: 10.1080/24701556.2020.1852429
26. Ranjani, S. Mohamed Sheik Meeran, S. Prakash, S. P. Mohammad, W. Kandasamy, R. and Srinivasan, H. (2020k). Multi potent aromatic nano colloid: synthesis, characterization and applications. AMB Express. 10(1):168. https://doi.org/10.1186/s13568-020-01104-5
27. Lakshya Mittal, Ranjani, S. Shariq Ahmed, M. Jeya Shree, T. Tahira Akther, Poompavai, S. Ignacio G. Camarillo. GowriSree, V. Raji Sundararajan. and Hemalatha, S. Turmeric-silver-nanoparticles for effective treatment of breast cancer and to break CTX-M-15 mediated antibiotic resistance in *Escherichia coli*. 2020 Inorg. Nano-Met. Chem. DOI: 10.1080/24701556.2020.1812644
28. Charannya S, Duraivel D, Padminee K et al. (2018) Comparative evaluation of antimicrobial efficacy of silver nanoparticles and 2% chlorhexidine gluconate when used alone and in combination assessed using agar diffusion method: An In vitro study. Contemporary Clinical Dentistry 9:204. doi: 10.4103/ccd.ccd_869_17
29. Ranjani, S. Pradeep, P. Vimalkumar, U, Ramesh Kumar, V and Hemalatha, S. Pungent antiinfective nanocolloids manipulate growth, biofilm formation and CTX-M-15 gene expression in pathogens causing Vibriosis. Aquaculture International.2021. 29:859–869
30. Shariq Ahmed M, Soundhararajan R, Akther T et al. (2019) Biogenic AgNPs synthesized via endophytic bacteria and its biological applications. Environmental Science and Pollution Research 26:26939-26946. doi: 10.1007/s11356-019-05869-6
31. Sah S, Rasool U, Hemalatha S (2020) Andrographis paniculata extract inhibit growth, biofilm formation in multidrug resistant strains of Klebsiella pneumoniae. Journal of Traditional and Complementary Medicine 10:599-604. doi: 10.1016/j.jtcme.2019.02.006
32. S R, M S, Adnan M et al. (2020) Synthesis, characterization and applications of endophytic fungal nanoparticles. Inorganic and Nano-Metal Chemistry 51:280-287. doi: 10.1080/24701556.2020.1784231
33. Rasool U, S P, Parveen A et al. (2018) Efficacy of Andrographis paniculata against extended spectrum β-lactamase (ESBL) producing E. coli. BMC Complementary and Alternative Medicine. doi: 10.1186/s12906-018-2312-8
34. Hasan M, Islam M, Islam M (2016) Phytochemistry, pharmacological activities and traditional uses of Emblica officinalis: A review. International Current Pharmaceutical Journal 5:14-21. doi: 10.3329/icpj.v5i2.26441

35. Tungmunnithum D, Thongboonyou A, Pholboon A, Yangsabai A (2018) Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. Medicines 5:93. doi: 10.3390/medicines5030093
36. Sabiha Sulthana, H. B. Ranjani, S. and Hemalatha, S. (2020) Comparison of efficacy of nanoparticles synthesized from leaves and flowers of *Russelia equisitiformis*, Inorg. Nano-Met. Chem. https://doi.org/10.1080/24701556.2020.1862218
37. Sai Nivetha, S. Ranjani, S. and Hemalatha, S. (2020) Synthesis and application of silver nanoparticles using *Cissus quadrangularis*, Inorg. Nano-Met. Chem, DOI: 10.1080/24701556.2020.1862219
38. Chouhan N (2018) Silver Nanoparticles: Synthesis, Characterization and Applications. Silver Nanoparticles - Fabrication, Characterization and Applications. doi: 10.5772/intechopen.75611
39. Wang L, Hu C, Shao L (2017) The antimicrobial activity of nanoparticles: present situation and prospects for the future. International Journal of Nanomedicine Volume 12:1227-1249. doi: 10.2147/ijn.s121956
40. Slavin Y, Asnis J, Häfeli U, Bach H (2017) Metal nanoparticles: understanding the mechanisms behind antibacterial activity. Journal of Nanobiotechnology. doi: 10.1186/s12951-017-0308-z
41. Sanchooli, N., Saeidi, S., Barani, H. K., & Sanchooli, E. (2018). *In vitro* antibacterial effects of silver nanoparticles synthesized using *Verbena officinalis* leaf extract on *Yersinia ruckeri, Vibrio cholera* and *Listeria monocytogenes*. *Iranian journal of microbiology*, *10*(6), 400–408.
42. Dakal T, Kumar A, Majumdar R, Yadav V (2016) Mechanistic Basis of Antimicrobial Actions of Silver Nanoparticles. Frontiers in Microbiology. doi: 10.3389/fmicb.2016.01831
43. Shah S, Gaikwad S, Nagar S et al. (2019) Biofilm inhibition and anti-quorum sensing activity of phytosynthesized silver nanoparticles against the nosocomial pathogen Pseudomonas aeruginosa. Biofouling 35:34-49. doi: 10.1080/08927014.2018.1563686
44. Singh M, Mallick A, banerjee M, kumar R (2016) Loss of outer membrane integrity in Gram-negative bacteria by silver nanoparticles loaded with Camellia sinensis leaf phytochemicals: plausible mechanism of bacterial cell disintegration. Bulletin of Materials Science 39:1871-1878. doi: 10.1007/s12034-016-1317-5

Tables

Due to technical limitations, table 1-4 is only available as a download in the Supplemental Files section.

Figures

Fig.1

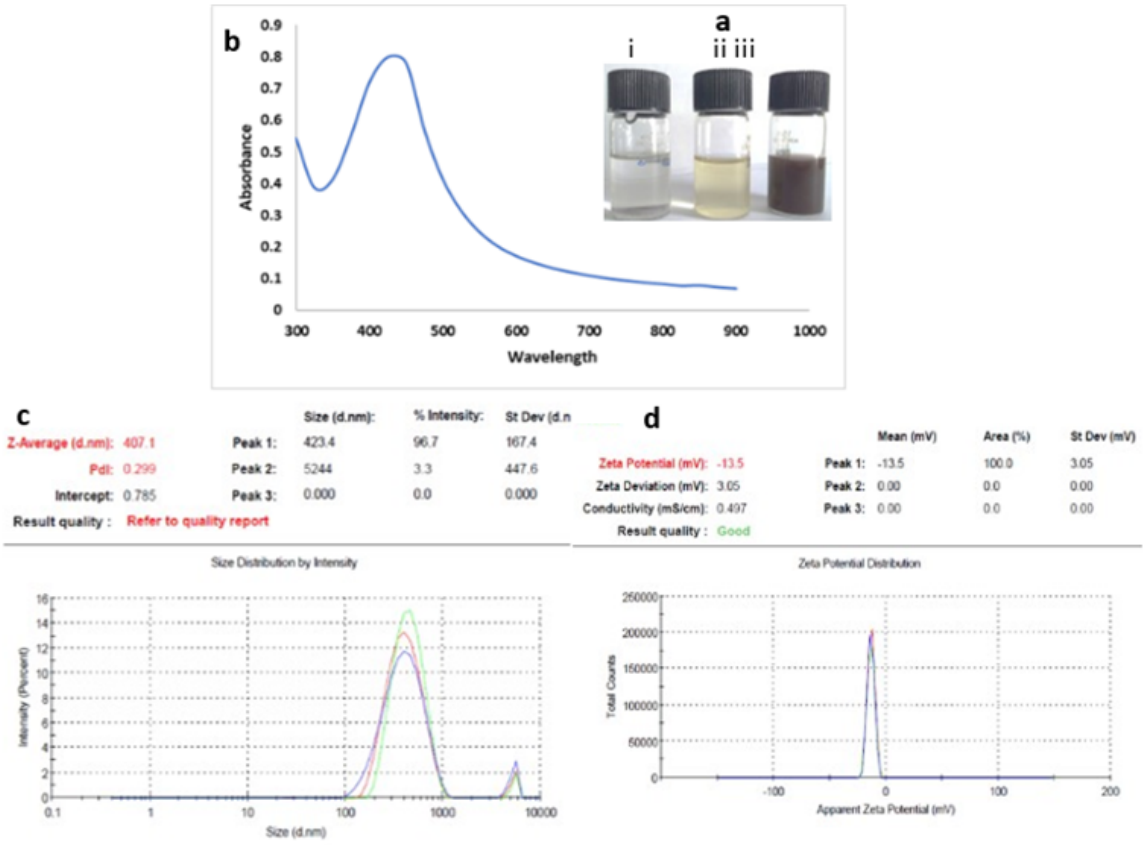

Figure 1

(Inlet fig a). i. 1mM silver nitrate solution, ii. Extract of Myrobalans , when mixed with 1mM silver nitrate solution, iii. Colour change during biological synthesis of green nanocolloid synthesis (MBNc) (b). Uv-Vis absorption spectra of Natural polymer mediated nanocolloid (MBNc) (c). Particle size distribution of MBNc, based on random motion of colloidal particle dispersed in medium (d). Zeta potential analysis of MBNc

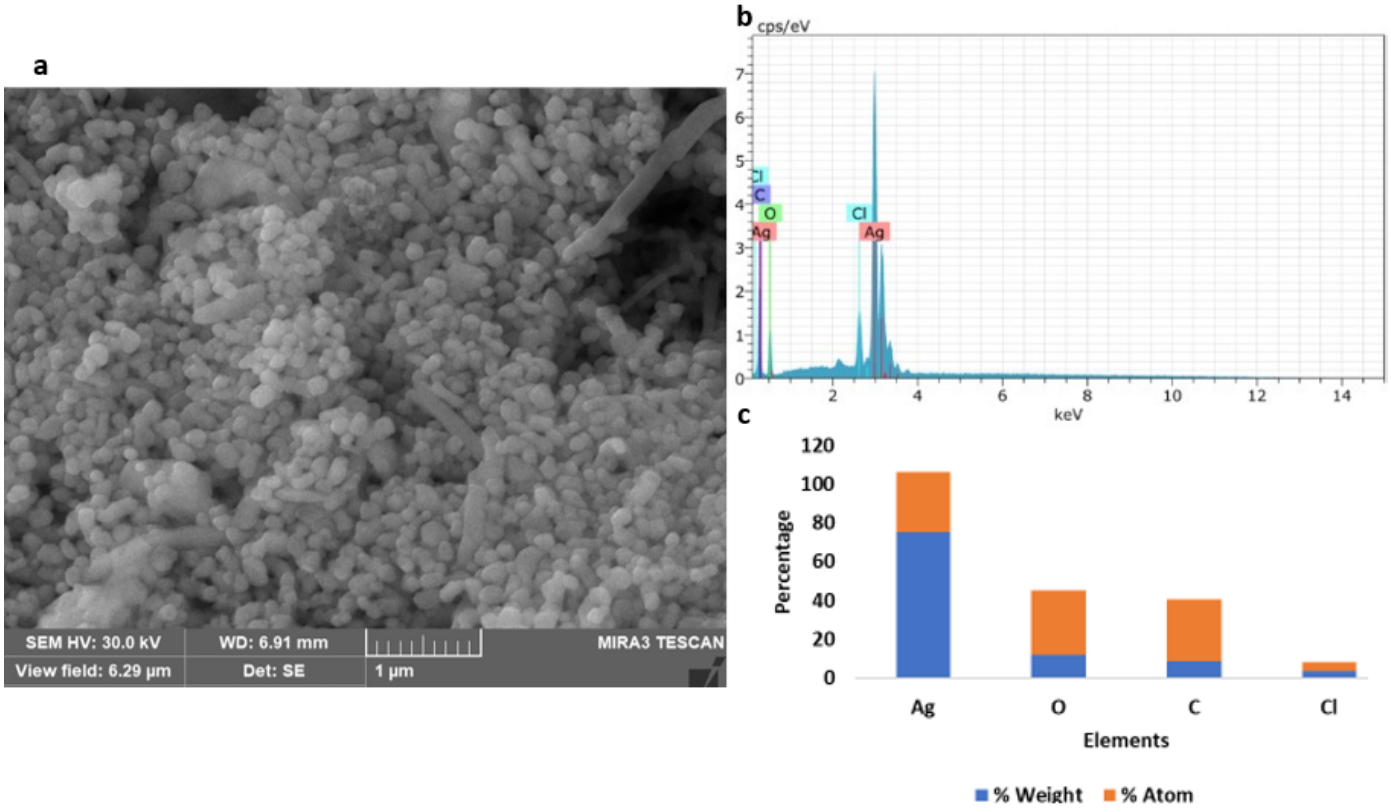

Figure 2

(a). Field emission scanning microscopic image of MBNc at 1µm scale (b). Qualitative and quantitative analysis of elements present in MBNc obtained through EDAX spectrum (c). Histogram showing the percentage atom & percentage weight of elements present in MBNc

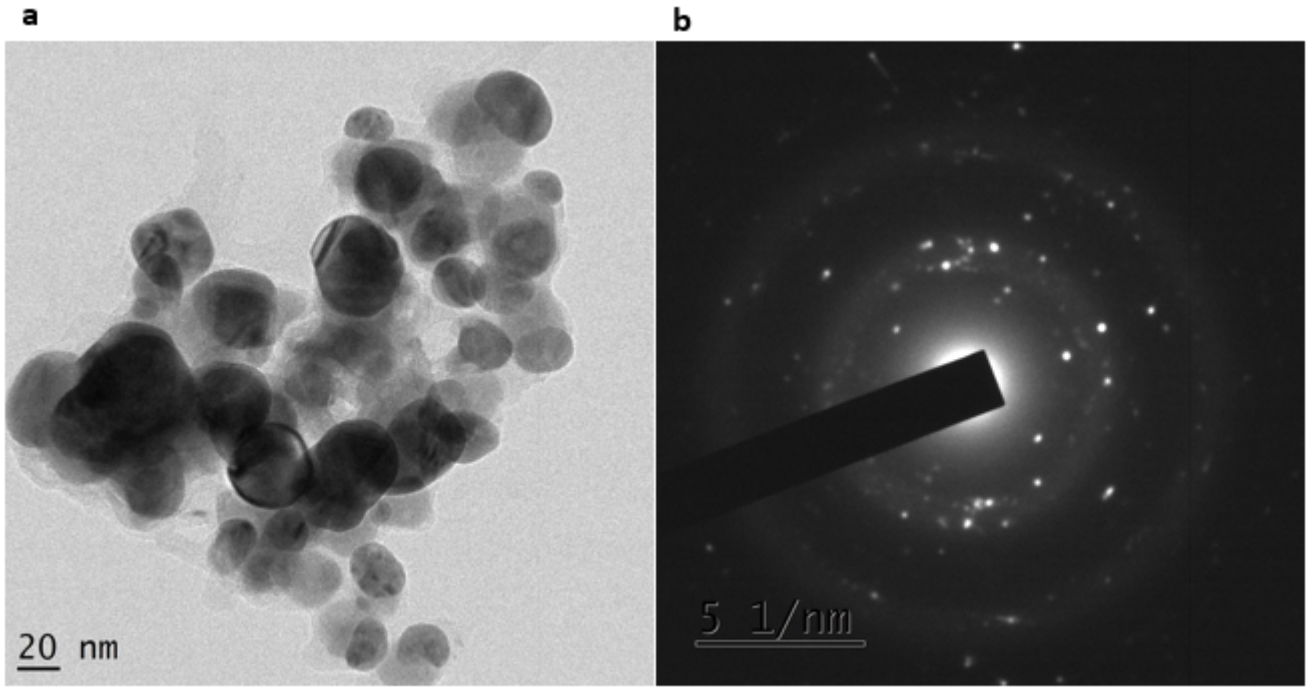

Figure 3

(a). HRTEM analysis of MBNc at 20nm scale (b) SAED pattern of MBNc shows polycrystalline nature of MBNc

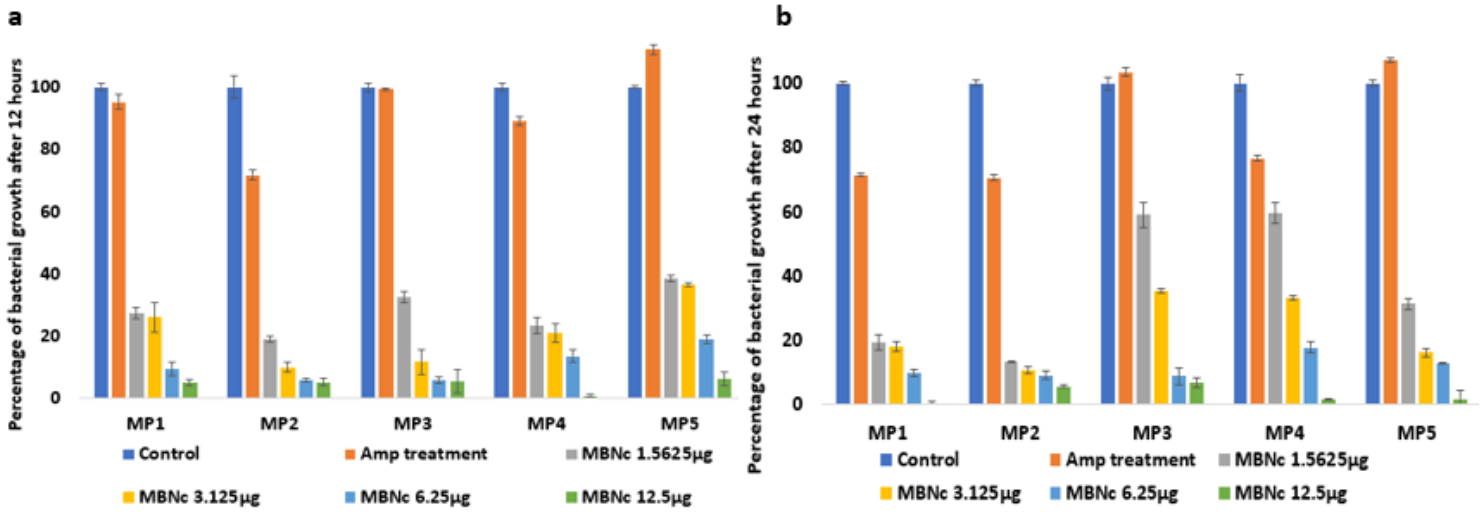

Figure 4

(a). Percentage of bacterial growth rate after 12 hours upon MBNc treatment. (b). Percentage of bacterial growth rate after 24 hours upon MBNc treatment.

Fig.5

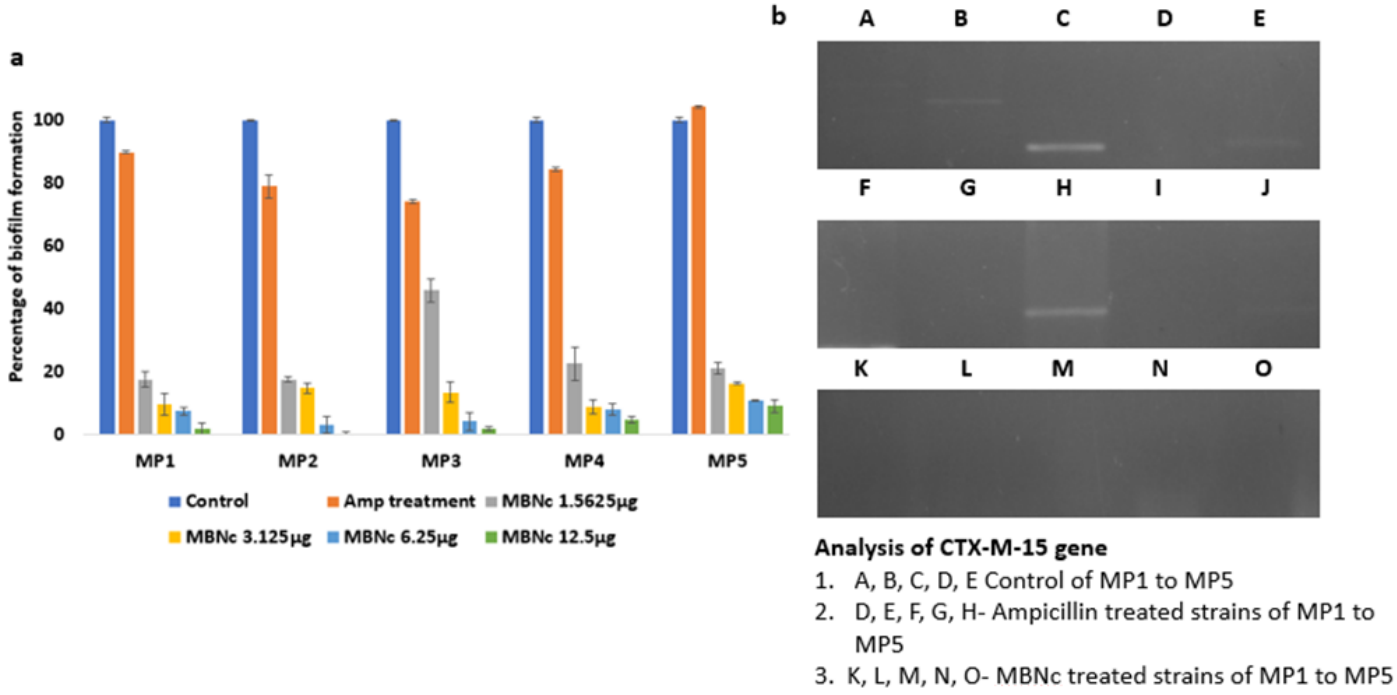

Figure 5

(a) Percentage of biofilm formation in marine pathogenic strains upon treatment with different concentrations of MBNc. (b). Analysis of CTX-M-15 gene: wells A, B, C, D, E- Control of V. harveyi (MP1), V. parahaemolyticus (MP2), V. alginolyticus (MP3), S. haemolyticus (MP4), S. haemolyticus (MP5), whereas only MP3 strain showed the presence CTX-M-15 gene. Wells F, G, H, I, J are Ampicillin treated strains, where there is downregulation of CTX-M-15 gene in MP3 strain. Wells K, L, M, N, O - Treated with MBNc, well M, MP3 showed complete abolishment of CTX-M-15 gene in V. alginolyticus (MP3) strain.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Tables.pdf