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Original Article

MORPHOLOGICAL IDENTIFICATION OF ENDEMIC MARINE SPONGE AND THE ANTIMICROBIAL ACTIVITY OF *ASSOCIATED BACTERIA*

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ABSTRACT

Objective: The primary objective is to morphologically identify a marine sponge from Sethubavachatram village in Tamil Nadu, India. Then to identify the bacteria associated with the sample to synthesize silver nanoparticles and to know antimicrobial property of the samples.

Methods: The sponges is identified by morphology and found to be *Hippospongia intestinalis*. The bacteria associated with the sponges is *Lysinibacillus* macrolides. Silver nanoparticles were synthesikzed and analyzed by SEM and TEM.

Results: The sponge that was recovered was identified using a number of sources that were derived from earlier publications. The sponge that was recovered was identified as *Hyattella intestinalis*

Conclusion: The antimicrobial assay confirmed that the synthesized particles are effective in inhibiting disease causing bacterial and fungal strains.

Keywords: Diversity, Marine sponges, Demospongiae, Sethubavachatram village, *Lysinibacillus* macrolides, Silver nanoparticles

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INTRODUCTION

Marine sponges, which are among the earliest multicellular creatures (metazoans), belong to the phylum Porifera [1, 2]. Spongiidae is a family of sponges that contains seven taxa with a wide range of forms and sizes, from low covering to erect gargantuan. The Spongiidae Gray, the 6 species have a thick main and secondary fibre skeleton and uniform skeletal fibres [3, 4].

In 1889, Lendenfeld published a paper on the taxonomy, morphology, and physiology of "horny sponges. Collagenous components, primarily spongin fibres, replace the mineral skeleton of horny sponges, converting to fibrillar collagen and reinforcing the sponge matrix [5, 6]. At the ordinal level, "horny" sponges are classed as Keratosa, Monoceratina, Dendroceratina, and Myxospongiae, Euceratosa, Dictyoceratida, and Dendroceratida. The three Dictyoceratida families are Dictyoceratida, Dendroceratida, and Verongida.

Fibres that are commonly cored and uncored, as well as primary and secondary fibres. They're pliable as well as compressible [7]. Internationally, 34 species have been depicted, with 24 of them being genuine. There have been roughly 14 Hyattella species found globally, according to earlier studies [8, 9].

Hyattella cribriformis, Hyattella intestinali, Hyattella meander Lendenfeld, Hyattella pertusa, Hyattella sinuosa, Hyattella tubaria Lendenfeld, and *Hyattella globosa Lendenfeld* are the seven *Hyattella* species found in India. *Hyattella* is found all throughout the world, especially in tropical oceans [10, 11]. Furthermore, there have been few reports from India, and the species described from the Indian Ocean have naming issues.

In this context, the main objective of this study was to identify the marine sponge *Hyattella intestinalis* from Sethubavachatram village in Thanjavur district, Tamil Nadu and also to identify the antibacterial potential of the nanoparticles synthesized via its associated bacetria [12].

MATERIALS AND METHODS

Collection and identification of *Hyattella intestinalis*

A marine sponge, *H. intestinalis*, was discovered in Sethubavachatram village [13] (fig. 1). Sethubavachatram is a village panchayat in the Thanjavur district of Tamil Nadu, India. Sethubavachatram is located at 10.2516118 latitude and 79.2775316 longitude. Chennai is the state capital of Sethubavachatram village. Sethubavachatram is a distance of 334.8 kilometres. All of the obtained samples were cleaned three times with tap water and twice with distilled water to remove any adhering associated animals [14].

With razor blades, the material was cut into small sections and kept. The structures were recorded using a Nikon SMZ 1000, NIS ELEMENTS version 4.20 (Nikon). Width of the main fibre and coring pattern were investigated. The secondary and tertiary fibre diameters were also measured using the same procedure. Diagnostic characteristics mentioned in the literature [6-9] were used to identify the sponges.

The Marine sponge was taxonomic identified using spicules obtained using nitric acid digestion and traditional identification keys [10]. A mix of literature and morphology was used to identify the recovered sea sponge. Shape, distribution of surface pores, colour, and surface ornamentation are among the morphological characteristics used to distinguish sponges. The organic skeleton's texture, structure, and composition are studied separately from the water canal system, as well as the inorganic skeleton's structure, composition, size, and geometry. In sponge taxonomy, a number of non-morphological traits have been found to be useful tools [11].

Making AgNPs outside of cells (Singh *et al.*, 2018)

In 100 ml of NB media, *Lysinibacillus macroides* were grown. The flask was kept in an orbital shaker at 120 rpm and 28 °C for one day. The medium was then spun in a centrifuge to get the supernatant. The supernatant was mixed with a filter-sterilized AgNO3 solution at a final concentration of 1 mmol. The mixture was then left to sit for 48 h at 120 rpm and 28 °C. During the synthesis, the colour of the culture medium was looked at to see if it changed. The AgNPs were separated by high-speed centrifugation at 12,000 g and 25 °C for 10 min, and then they were washed well with water to get rid of any metal ions that hadn't been changed and any other parts. The cleaned nanoparticles were dried by air and turned into a powder.

Analysis by FTIR and UV-visible spectroscopy

The silver nanoparticles were looked at with a UV and visible spectrophotometer. The silver nanoparticles were scanned with a Perkin Elmer Spectrophotometer between 200 and 1000 nm to find the characteristic peaks. The FTIR analysis was done with a Spectrophotometer system, which was used to find the characteristic peaks between 400 and 4000 cm-1 and the functional groups they belong to. It was written down what the UV and FTIR peaks were. Each analysis was done twice to make sure the spectrum was correct.

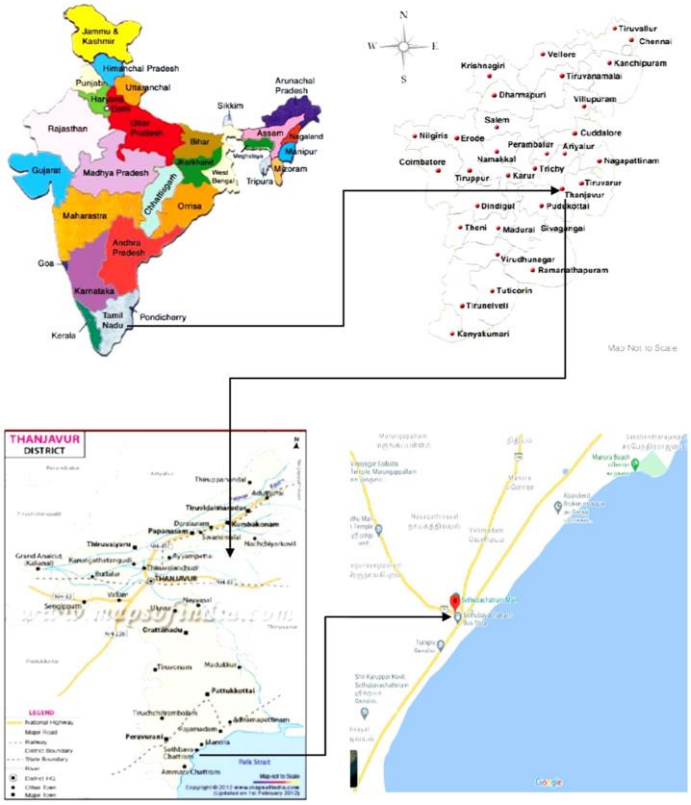


Fig. 1: Location of marine sponge collection

Electron microscopy and EDX

The ZEEISS-SEM machine was used to look at the size and shape of nanoparticles. Silver nanoparticles that had already been dried were sonicated with distilled water, and then small droplets of silver nanoparticles were put on a glass slide and left to dry. The ZEEISS-SEM machine was run at about 10-5 torr of vacuum. The voltage to speed up is 10 kV. At a working voltage of 80 kV, CM30–Philips did the TEM study. Energy-dispersive X-ray spectroscopy (EDS), which was attached to the SEM, was used to fig. out what the sample was made of. The SEM machine did the EDX test on the Ag sample.

X-ray diffraction

Cu Ká radiation was used with an X-ray diffraction technique (Philips PAN analytical, The Netherlands) to study the change in phase of both calcined powder and sintered samples. The generator was set to have a voltage of 35 KV and a current of 25 mA. In continuous scan mode, the Ag samples were scanned in the 2è range from 15 to 700oC. The rate of scanning was 0.04/s.

Analysis of antimicrobial activity

Preparation of the medium

In a container that holds 1000 millilitres of deionized water, disperse 39.0 grammes of PDA. Bring the medium up to a boil, then completely dissolve the ingredient. Autoclave for fifteen minutes at a pressure of 15 Ibs and a temperature of 121 C. When an application calls for a pH of 3.5, acidifying the medium using sterile tartaric acid 10 percent will provide the desired results. It is necessary to use about one cc of acid for every one hundred milliliters of sterile, refrigerated medium. After adding the acid, you should not heat the medium [21].

Microorganisms

For the bacterial experiments, Escherichia coli (MTCC 732), Pseudomonas aeruginosa (MTCC 1035), and Staphylococcus aureus (MTCC 3160) were utilised. For the fungal assays, Candida albicans (MTCC 183) and Aspergillus niger (MTCC 1783) were employed. The Microbial Type Culture Collection (MTCC) at the Institute of Microbial Technology (IMTECH) in Chandigarh, India, is where one may get their hands on these cultures.

The preparation of pure culture for a period of twenty-four hours. Within a Roux bottle that contained about 10 milliliters of physiological saline, the bacteria were suspended. Every one of them was then streaked on the proper culture slants before being placed in an incubator at a temperature of 37 °C for a period of 24 h. The only exception was the fungal culture, which was placed in an incubator at a temperature of 25oC for a period of 48 h. After the incubation time came to an end and growth was seen, the tubes were kept at a temperature between 2 and 8 degrees Celsius until they were used.

Performing preparatory work on the experimental sample solutions

Together with two standard solutions, 10 mg of silver nanoparticles (AgNPs) were dissolved in 10 ml of a solvent called deionized water. The standard solutions were chloramphenicol (25 mg/ml distilled water) for bacteria and fluconazole (25 mg/ml distilled water-thirty litres) for fungus. They were maintained in a cold atmosphere up to the point when they were needed for the experiment [22].

Discs of filter paper are prepared for the drying process

By putting them in the oven, four Whattman filter paper No. 1 discs with a diameter of 6 mm each are sterilized by the hot air produced there. After the sample was sterile, 50, 100, and 150 l of it were placed onto discs, and 30 l of a standard solution containing chloramphenicol and fluconazole was used to compare the test solution to the other solution. They were maintained in a cold atmosphere up to the point when they were needed for the experiment.

Antimicrobial assay

The procedure known as disc diffusion was used to generate an antibiogram using the samples (NCCLS, 1993; Awoyinka *et al.*, 2007) Petri plates were prepared with a volume of 30 ml of NA/PDA medium. Inoculation of the test organism was performed on a plate of hardened agar using a micropipette. After inoculation, the plate

was distributed and allowed to dry for ten minutes. On the surfaces of the medium, bacteria that had been cultured in broth were distributed evenly. In order to inoculate the Nutrient agar and PDA plates in a manner that is consistent, a sterile cotton swab is first submerged in a microbiological test solution that has been standardized. Inoculums that contained different microbial strains were spread onto plates made of nutrient agar and PDA. On the surface of an infected agar plate, sterile filter sheets with a diameter of 6 mm and containing 50, 100, and 150l of AgNPs, Standard solutions as Chloramphenicol, and Fluconazole 30l were inserted using sterile forceps. The plates containing the bacterial strains were kept in an incubator at 37 degrees Celsius for 24 h, while the yeast strains were kept there for 48 h. Every sample was looked at three separate times.

Conducting an analysis of the inhibition zone

The antibacterial potential of test compounds was determined by using the mean diameter of the zone of inhibition that surrounded the disc in mm as the foundation for the calculation. The zones of inhibition of the microorganisms under study that were caused by the chemicals were measured on a mm scale.

RESULTS AND DISCUSSION

Identification of *Hyattella intestinalis*

The sponge that was recovered was identified using a number of sources that were derived from earlier publications [12-14]. The sponge that was recovered was identified as *Hyattella intestinalis*.

Hyattella intestinalis was formerly known as Spongia intestinalis, which means "intestinal sponge." It was eventually determined to be caused by *Hyattella intestinalis* after a sequence of genetic changes was discovered. For the sponge he discovered in the East Indies, Carter called it Hircinia clathrata in 1881. It was then renamed Cateriospongia clathrata and Hyattella murrayi, Lendenfeld 1889, after Carter's discovery in the Gulf of Mannar environment.

Material examined

1. *Hyattella intestinalis* 1: CMFRI. DNR No. BA.1.1.2.9 (fig. 1) Muttom, Kanyakumari, Tamilnadu (Lat: 8°7'20.07"N, Long: 77°18'45.63"E), 10.11.2018, Gillnet sample (No. MT 53) deposited in the Museum of National Biodiversity Repository, CMFRI, Kochi, India.

2. *Hyattella intestinalis* 2: CMFRI. DNR No. BA.1.1.2.9.2 (fig. 2) Muttom, Kanyakumari, Tamilnadu (Lat: 8°7'20.07"N, Long: 77°18'45.63"E), 10.11.2018, Gillnet sample (No. MT 71) deposited in the Museum of National Biodiversity Repository, CMFRI, Kochi, India.

3. *Hyattella intestinalis* 3: CMFRI. DNR No. BA.1.1.2.9.4 (fig. 3), Muttom, Kanyakumari, Tamilnadu (Lat: 8°7'20.07"N, Long: 77°18'45.63"E), 10.11.2018, Gillnet sample (No. MT 50), deposited in the Museum of National Biodiversity Repository, CMFRI, Kochi, India.

Description

Sponge measures 8 cm in height and 6 cm in breadth and has a cylindrical hollow fiber body. The finger height is between 3-6 cm and the thickness is between 0.1-0.3 cm. The lamella has a width of 0.8 cm and stiffness of 0.2 cm. Tubular in shape, the hollow body has branching ends on each side. The surface of the sponge is unevenly aerated, and the sponge has erect branching with branch lengths varying from 2 to 6 cm. They adhere readily to the substrate's surface. Consistently distributed oscules, typically on the tip of the sponge digits, are found evenly distributed around the surface, creating the appearance of a porous reticulated surface. Oscules range in diameter from 0.1 to 1.0 cm. Ostia is a little fungus that grows on the skin's surface and can be transmitted from person to person. The consistency is robust, compressible, and difficult to rip, and the digitiform surface processes, like the overall shape, are broad and extensively tapered. In this investigation, the morphological identification of Hyattella intestinalis was performed and compared to the available literature.

Skeleton

A well-developed fibre network consists of main, minor, and tertiary fibres that are all linked. The main skeleton is formed mostly of reticulations of sponge fibres, which are often organised into networks with homogeneous fibre cross-sections. The parasitic worm *Hyattella intestinalis* dwells in the intestines and feeds on the host's blood. Primary fibres with diameters ranging from 39.33-52.0 m are shown in fig. 2, which are cored with minute sand particles. Secondary fibres are those that lack a core and measure 14.05 to 21.9 m in thickness (fig. 2C). Tertiary fibres varies between 7.3 and 11.28 m. Fig. 2 illustrates the identification of the fiber-reinforced skeleton.

Fig. 2: Morphological identification of *Hyattella intestinalis*

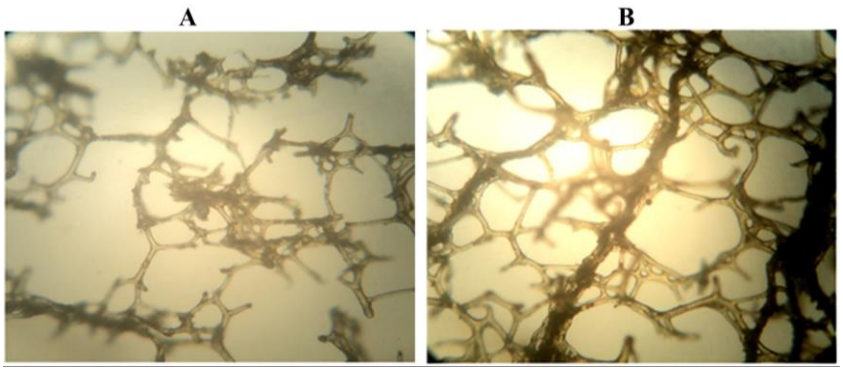


Fig. 3: Identification fibre skeleton-(A) Cross section and (B) longitudinal section of *Hyattella intestinalis*

Taxonomy

Phylum: Porifera Grant

Class: Demospongiae Sollas

Subclass: Keratosa Grant

Order: Dictyoceratida Minchin

Family: Spongiidae Gray

Genus: *Hyattella*

Species: *intestinalis*

Remarks

At least three type species have been identified: Hippospongia intestinalis (Lamarck, 1814), USNM 7689 (holotype), Smithsonian National Museum of Natural History (Indian Ocean), Spongia intestinalis (Lamarck, 1814), MNHN DT (Holotype) 584, Musee National d' Histoire Naturelle, Paris (Indian Ocean), and Hippospongia clathrate BMNH 1920.12.9.46 (Holotype), British Museum of Natural History, (locality: Indian Ocean) were reported during 1800s.

Making AgNPs outside of cells

The first reaction mixture (yellow) and the 48-hour mixture (Deep brown).

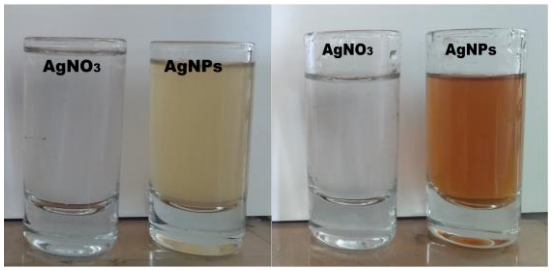


Fig. 4: Extracellular synthesis of AgNPs

$AgNO_3$: 1 mmol $AgNO_3$ without Lysinibacillus macrolides supernatant

After 48 h of incubation, the supernatant from AgNPs (1 mmol AgNO3) with Lysinibacillus macrolides (Brown Deep colour). The extracellular synthesis of nanoparticles was seen to be happening when the reaction mixture turned a different colour. Within 48 h, the colour of the supernatant changed from yellow to a deep brown (fig. 4). As the Ag0 level went down, the colour got darker as time went on. Control (without bacteria) didn't change colour when put in the same conditions for the same amount of time.

Ultraviolet and visible spectrometric analysis

Most people know that UV–Vis spectroscopy can be used to look at nanoparticles in aqueous suspensions whose size and shape can be controlled. Fig. 5 shows the UV-Vis spectra that were taken from the reaction medium 48 h after the reaction started. The peak at 420 nm in the UV–Vis spectra of the reaction mixture of silver nitrate solution and Lysinibacillus macrolides supernatant showed that silver nanoparticles were present. By looking at the UV–vis spectrum of the reaction mixture, it was possible to track the change from pure Ag+ions to Ag0.

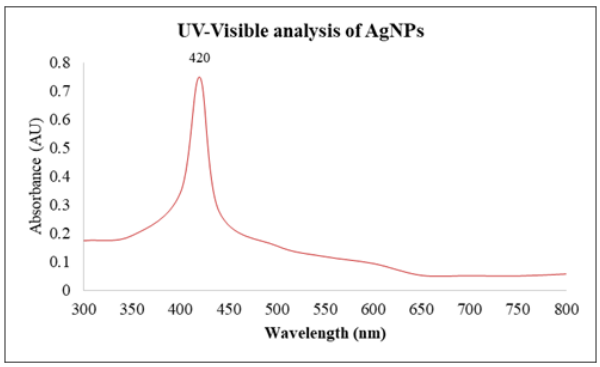


Fig. 5: Analysis of the UV-Vis spectrum of AgNPs

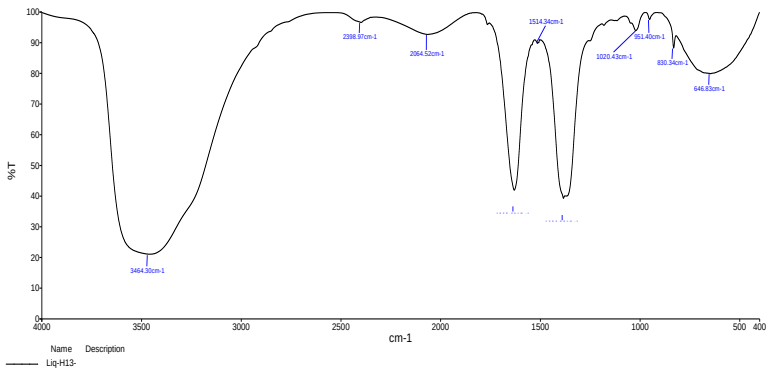


Fig. 6: Analysis of AgNPs by fourier transform infra-red spectral

Table 1: Analysis of AgNPs by fourier transform infra-red spectral

Frequency (cm-1)	Bond	Functional group
3464.30	O-H stretch, H-bonded	Phenols
1630.45	N-H bend	1 ° amines
1514.34	N-O asymmetric stretch	Nitro compounds
1020.43	C-N stretch	Aliphatic amines
951.40	=C-H bend	Alkanes
830.34	C-Cl stretch	Alkyl halides
646.83	C-Br stretch	Alkyl halides

Fourier transform infrared spectrum analysis of AgNPs

The FTIR spectrum of silver nanoparticles was looked at to find the likely biomolecules that stabilised and capped the silver nanoparticles made by Lysinibacillus macrolides supernatant. The peaks could be seen (fig. 6 and table 1).

Analysis of the shape of AgNPs

Morphological analysis of AgNPs was done with SEM, TEM, analysis to look at the shape, texture, and chemical make-up of the nanoparticles. SEM and TEM (scanning electron microscope and transmission electron microscope) analysis of AgNPs

SEM analysis was done to fig. out the shape and size of the NPs. The results showed that higher density polydispersed spherical NPs of different sizes, ranging from 51 to 72 nm, were made, as well as that the nanoparticles were cubic and crystalline (fig. 7). When looked at with a SEM, most of the nanoparticles were clumped together, and only a few were spread out.

The size of the nanoparticles ranged from 48.04 to 113.95 nm, and the average particle size was found to be 73.51 17.65 nm (fig. 8). This was found by plotting the data into histograms to study the particle size distribution. Overall, most of the NPs were between 50 nm and 70 nm in size, which shows that they were made with a size less than 100 nm (NPs 100 nm) (fig. 9). SEM and TEM analyses were done to fig. out the size and shape of the AgNPs. The results showed that the AgNPs had a complex density and were made up of spherical particles of different sizes.

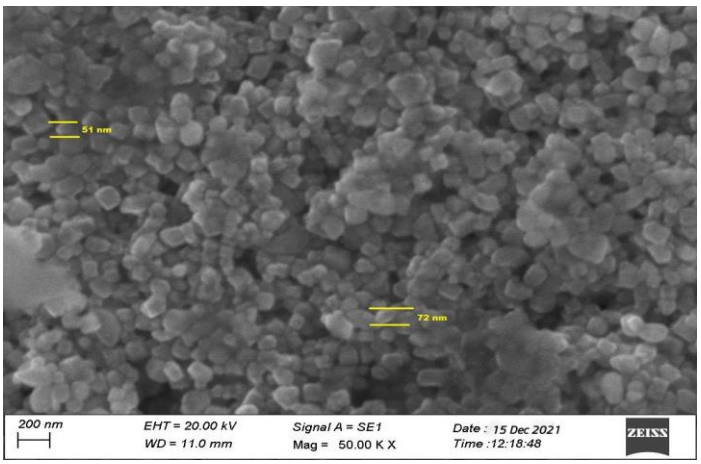


Fig. 7: Analysis of nanoparticles by scanning electron microscopy (SEM), The dynamic light scattering measurement was done to find out how big the nanoparticles were. Laser diffraction showed that the size of the particles was between 40 and 120 nm

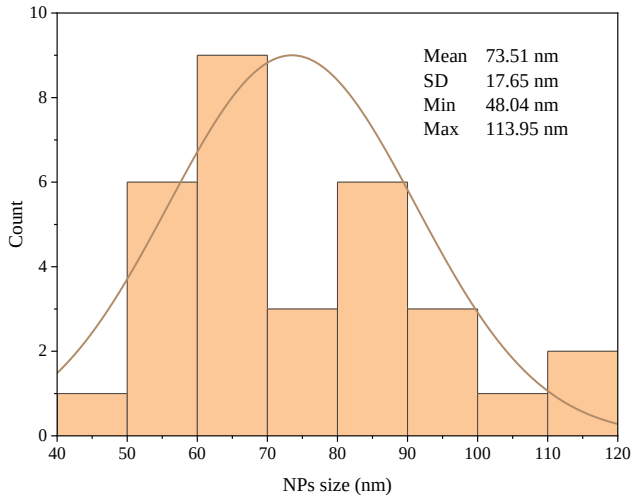


Fig. 8: This histogram shows how the sizes of NPs vary

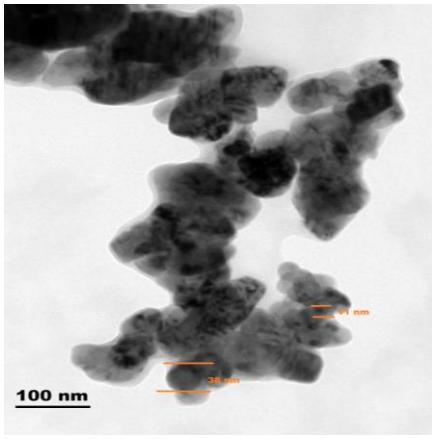
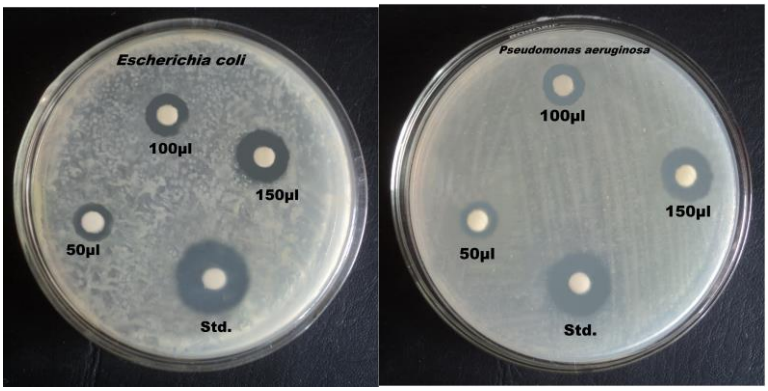
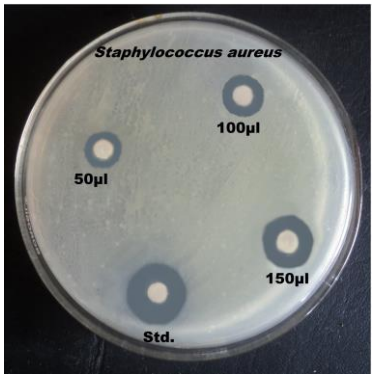


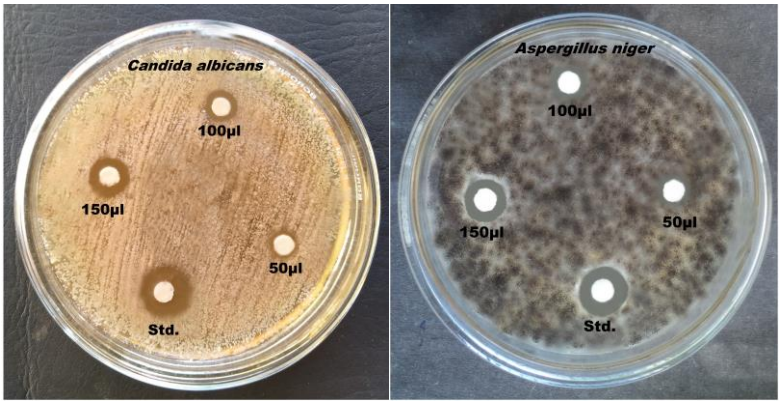
Fig. 9: Transmission electron microscopic (TEM) image of silver nanoparticles with Cluster in the range between 11 to 38 nm



Escherichia coli **Pseudomonas aeruginosa**



Staphylococcus aureus



Candida albicans **Aspergillus niger**

Fig. 10: Plate 1-Antimicrobial activity of AgNPs

Table 2: Antimicrobial activity of AgNPs against microbial strains

Microbial strains	AgNPs concentration			Std. (30 µl)
	50 µl	100 µl	150 µl	
Bacterial strains				
Escherichia coli (mm)	3.50±0.24	6.65±0.46	10.70±0.74	13.45±0.94
Pseudomonas aeruginosa (mm)	2.90±0.20	6.05±0.42	10.15±0.71	12.80±0.89
Staphylococcus aureus (mm)	3.20±0.22	6.35±0.44	10.25±0.72	12.50±0.87
Fungal strains				
Candida albicans (mm)	2.30±0.16	4.75±0.33	7.55±0.52	11.25±0.78
Aspergillus niger (mm)	2.10±0.14	4.30±0.30	7.40±0.51	11.05±0.77

Values expressed as mean±SD; Standard: Chloramphenicol and Fluconazole; mm: Millimeter.

The antimicrobial activity shows that the synthesized nanoparticles are effective against bacterial and fungal strains (fig. 10). The particles can be formulated into drugs as it inhibits the growth of disease-causing pathogens (Escherichia coli, Pseudomonas aeruginosa and Staphylocaoccus aureus) (table 2). The carcinogenic fungal pathogens Candida albicans and Aspergillus niger also inhibited and the particle shows potential effect (fig. 10).

DISCUSSION

This study compared a longitudinal and cross-sectional slice of Hyattella intestinalis to previously reported data [12]. This species is reported to occur in the Gulf of Mannar, Palk Bay, Mandapam, Vedalai, Shingle Island, Adiaman beach, Manali Island15, and Muttom in the Indian state of Tamil Nadu. Adithirampattinum (present study) is a town located in Tamil Nadu, India. Hippospongia intestinalis samples were found in the Gulf of Mannar and Ceylon waters and exhibited features such as an elongated tubular shape with perforated walls, a conulose surface, and an irregular amber-colored skeleton. These features have been detected in Hippospongia intestinal is samples collected from the Gulf of Mannar and Ceylon. The species Hippospongia clathrata (=Hyattella intestinal is) was renamed Hippospongia intestinalis when it was discovered in Okha (Gujarat, India) in the Northern Arabian Sea. The current sample has been found to correlate to both the original description of Hyattella intestinal is and three subsequent findings in the Gulf of Mannar Ecosystem. All of these investigations observed features such as an elongated tubular form with perforated walls, amber-coloured fibres, an uneven, conulose and minutely conulose surface, terminal oscules, and a rough feel [11, 12].

Samples of flat tidal sediment from the sea samples were used to find a Gram-positive, rod-shaped, endospore-forming bacterium called strain BLB-1(T) [13, 16]. Because they are very active, bacteria that live on red algae could be a source of bioactive secondary metabolites [17].

Lysinibacillus varians supernatant can be used to make stable silver nanoparticles outside of cells [15, 18]. Observing a change in colour is used as a way to screen microbial isolates for making silver nanoparticles [23]. Surface plasmon resonance was said to be the reason why the colour changed from pale yellow to dark brown while producing silver nanoparticles [16].

Nanoparticles made of noble metals have SPR bands that show up in the visible range. Green methods were compared to chemical methods, and the data was looked at using UV-visible spectroscopy, SEM, EDX, TEM, AFM and FT-IR [14, 17]. This method can make nanoparticles less dangerous, make them better for the environment, lessen their side effects, and lower the cost of making them [14]. The surface plasmon resonance (SPR) of silver was found at 420 nm in the UV–vis absorption spectrum [20]. This was thought to be the SPR band of AgNPs. It is known that SPR in nano-silver causes an absorption band at about 400–420 nm. When tested with Lysinibacillus sphaericus, the average size of the AgNPs was between 14 and 21 nm. FTIR spectra showed that the tops of the synthesised AgNPs were made of nitrogen-containing biomolecules [19].

SEM showed that the average size of the nanoparticles was 73.51 17.65 nm, while TEM showed that their sizes ranged from 11 to 38 nm and that they were both round and crystalline. The size of silver nanoparticles was found to be between 10 and 20 nm by TEM when using the green synthesis method and extracellular production of silver nanoparticles usin the same species [18]. When looked at with TEM, most of the nanoparticles gathered together and not many were spread out (fig. 9). AgNPs exhibited activities that were not or slightly cytotoxicity [20].

Silver nanoparticles are just as effective against both Gram-positive and Gram-negative bacteria, which can be inferred. Several other studies have also shown that AgNPs kill both Gram-negative and Gram-positive bacteria (such as E. coli, S. aureus, B. subtilis, S. mutans, and Staphylococcus epidermidis) [23]. Zetapotential measurement confirmed that the silver nanoparticles that were made were negatively charged. By reducing metals, these negatively charged AgNPs may stop Gram-negative bacteria from growing [23-25].

CONCLUSION

Considering the morphological similarities, the present species from Sethubavachatram (Bay of Bengal) might be *Hyattella intestinalis*. Further, characterization and confirmation studies are warranted in future. The bacteria associated with the sponges is found to be Lysinibacillus macrolides. Lastly, this study offered a simple, highly effective, eco-friendly, and cheap way to make AgNPs that are bioactive in more than one way.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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