

Biosynthesis of Silver Nanoparticles by Marine *Micromonospora* species (KU 867645) with Antibacterial Activity against Multidrug-resistant Hospital-acquired Uropathogens

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Mahroop and Ahmed: Biosynthesis of Silver Nanoparticles by Marine *Micromonospora* sp.

Multidrug resistance is an emerging major clinical problem rapidly enhancing throughout the world, which require novel effective drugs to control this problem. The present study was aimed at investigation of silver nanoparticles synthesized by *Micromonospora* species for antibacterial activity against hospital-acquired uropathogens. Marine actinomycetes were collected from Kayalpatnam, located at Tuticorin, Tamil Nadu, India. Totally, six actinomycetes were isolated and identified based on their spore formation and biochemical studies. Six isolates were belonged to the genera of *Micromonospora* species. Among the six, *Micromonospora* species, KPMS10 showed potent antibacterial activity against Gram-negative and Gram-positive uropathogens. Silver nanoparticles synthesized by *Micromonospora* species KPMS10 were isolated, characterized using ultra violet-visible spectroscopy, Fourier transform infrared spectroscopy and scanning electron microscopy. The shape of nanoparticle was found to be spherical with an average size of 80 nm. The antibacterial activities of silver ion against uropathogens were found to be superior to third generation cephalosporins. These silver nanoparticles were found to be least haemolytic at 20 µg/ml. Biosynthesis of silver in combination with actinomycetes would be effective against clinical multidrug-resistant pathogens.

Key words: Multidrug, hospital infection, silver, haemolytic

Hospital-acquired infections are emerging over the past few decades, which are the cause of high morbidity and mortality of hospitalised patients. These infections has become a global health problem as mechanism for resistance has been reported for all currently available antimicrobial agents^[1]. Most hospital deaths are directly or indirectly linked to hospital-acquired infections and estimated as 80%^[2]. In India, 10-30% patients are admitted in hospitals or nursing homes are associated with hospital-acquired infection. This optimism regarding the new chemical compound was soon shattered as report on drug resistant bacteria was arisen shortly in associated with hospital infections and development of drug resistant organisms and the rate is 3.4%^[3]. As new antibiotics were discovered and taken in to use, the bacteria responded by developing various resistant forms.

The use of antimicrobials has been increased and the resistant mechanism by pathogenic bacteria also increased in complexity^[4]. Microbes can pose a threat

in hospital environments where patients with weakened immunity are at increased risk for contracting hospital-acquired infections^[5]. Arrest of the spread of drug resistant bacteria with effective treatment is yet to be established. Thus, endeavours for the discovery and application of nanotechnology are the need of the hour. Biosynthetic silver nanoparticles (AgNPs) using bacteria, fungi and plants have already been well documented^[6-8]. Indeed over the past several years, AgNPs were synthesized by a potential new antibiotic developer, actinomycetes from marine sediments^[9]. Actinomycetes are autochthonous free living Gram-positive saprophytic marine micro flora and can be isolated from marine sediments. The obligate marine

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actinomycetes represent a major source for production of numerous natural biological metabolites especially antibiotics^[10]. AgNPs play an important role in controlling bacterial infections^[11,12]. Silver is the metal of interest as it provides the most durable antimicrobial protection against microorganism. The mechanism of silver resistance offered by bacteria using a silver binding protein is well documented^[13].

In addition to nanoparticle synthesis, the broad spectrum antimicrobial activity of AgNPs was studied against clinical pathogens^[14]. AgNPs have been the most promising source to kill microbes very effectively because they act both intra and extracellularly. AgNPs act as a strong bactericidal agent and depict activity against both multidrug-resistant Gram-positive and negative bacteria^[15,16]. Marine actinomycetes would constitute a valuable resource for novel metabolites of pharmaceutical and medicinal interest, including antimicrobial agents^[17]. The objective of this work was to synthesize, characterize and evaluate the efficacy of biosynthesized AgNPs against hospital-acquired uropathogens.

MATERIALS AND METHODS

Sample collection:

Systematic screening of actinomycetes from marine sediments was done by random sample collection at 200 m distance and at a depth of 5 m using core samples from Gulf of Mannar, East coastal region, Kayalpatnam, Tuticorin district, Tamilnadu, India. The central portion of the marine sediments were aseptically transferred to the sterile bottles during June 2013 and brought to the laboratory covered in an ice bag. The sediment sample was blackish brown colour and of a sandy texture.

Isolation of actinomycetes:

The marine sediments were air-dried to minimize bacterial contaminants. One gram of sediment was serially diluted up to 10^{-6} dilution. About 1 ml of the diluted sample was permitted in to the Petri plate followed by actinomycetes isolation agar (AIA) medium supplemented with cyclohexamide 50 µg/ml and nystatin 50 µg/ml. After solidification, all the plates were incubated at 28° for 7-15 d until the colonies were developed. All the isolates were identified by slide culture method. A drop of 0.1% trypan blue stain was placed over the glass slide and then cover slip was placed over the stain gently. The slide was examined under bright field microscope and the spore was noted.

Biochemical characterization of actinomycetes:

After preliminary studies, the isolates were identified by biochemical analysis i.e. indole production, methyl red and Voges-Proskauer test, citrate utilization test, catalase test, oxidase test, starch hydrolysis, casein hydrolysis and urea hydrolysis were studied.

Analyses of multiple antibiotic resistance (MAR) index:

According to the guidelines of the Clinical and Laboratory Standards Institute, multidrug resistance was detected by using disc diffusion test, which was performed on Muller-Hinton agar (MHA) medium. The test bacteria were inoculated using the swab method. After 10-15 min, selected antibiotic discs were placed over the bacterial growth at equal distance. The plates were incubated at 37° for 24 h. After incubation, the zone of inhibition was considered as an indicator of drug resistance to the bacterial growth. MAR index was calculated by using following Eqn., MAR index = number of antibiotics to which the isolate was resistant/ total number of antibiotics tested.

Antibacterial activity of secondary metabolites:

Antimicrobial activity of *Micromonospora* species KPMS10 culture filtrate was analysed by agar well diffusion method. The 24 h nutrient broth culture of multidrug-resistant pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus* sp., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *P. vulgaris* were isolated from hospital-acquired infection and bioassayed against standard antibiotics. About 100 µl of culture filtrate was used as a test sample against clinical isolates. Inoculated plates were incubated at 37° for 24 h. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

Biosynthesis and characterization of AgNPs:

The isolated *Micromonospora* species KPMS10 strain was inoculated in to International *Streptomyces* Project medium IV (ISP IV) and incubated at 28° for 7-15 d. The culture broth medium was centrifuged at 5000 rpm for 10-20 min to remove cell debris. About 10 ml of cultured filtrate was permitted in to 100 ml of 1 mM AgNO₃ solution and incubated at room temperature in a shaker under dark condition. The bioreduction of pure AgNO₃ solution was monitored at regular intervals using a UV/Vis spectrophotometer between 190-1100 nm. Synthesized AgNPs were collected by

subsequent high speed centrifugation at 15 000 rpm and the functional group was detected by Fourier transform infrared spectroscopy (FTIR) between 4000-400 cm^{-1} . Morphology and size of AgNPs was studied under scanning electron microscope (SEM).

Antibacterial activity of AgNPs:

Antimicrobial activity of the AgNPs was evaluated using agar well diffusion method. Multidrug-resistant pathogens such as *E. coli*, *K. pneumoniae*, *Enterococcus* sp., *S. aureus*, *P. aeruginosa*, *P. mirabilis* and *P. vulgaris* were isolated from hospital-acquired infection and inoculated into the nutrient broth and incubated at 37° for 24 h. After incubation, test pathogens were inoculated on MHA using sterilized cotton swabs. In each of these plates, wells were cut using a sterile gel borer and 50 μl of AgNPs were placed against clinical isolates. Inoculated plates were incubated at 37° for 24 h. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

Haemolysis assay of AgNPs:

The cytotoxic effect of AgNPs was studied using the haemolytic test^[18]. A pellet of RBC was collected and washed three times with phosphate-buffered saline (PBS) at 10 000 rpm for 3 min. The obtained RBC was diluted with PBS at 1:10 ratio. About 400 μl of RBC suspensions were treated with 100 μl of nanoparticles in Tyrode at 1, 5, 10, 25 and 50 $\mu\text{g/ml}$ and Triton X-100 as positive control. The suspension was incubated at room temperature by shaking the plate for 24 h. The percent haemolysis was calculated by following Eqn., $H (\%) = (\text{sample OD} - \text{Tyrode OD}) / (1\% \text{ Triton X-100 OD} - \text{Tyrode OD}) \times 100$.

Phylogenetic analysis:

The 16S rRNA sequences of *Micromonospora* species KPMS10 was blasted and deposited in the Genbank of National Centre for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

The present investigation involved the synthesis of AgNPs by the marine actinomycetes. Based on cultural characterization, the actinomycetes used were identified as *Micromonospora* species. All isolated *Micromonospora* strains were Gram-positive but differed morphologically by producing different mycelia and spores. *Micromonospora* species showed

monospore on aerial mycelium. The colors of the substrate mycelium were yellowish white to dark ash after sporulation. The morphological characteristics of these isolates were consistent with their classification in the genus *Micromonospora*^[19]. The *Micromonospora* species KPMS10 was strictly found to be positive in indole, Voges-Proskauer, catalase, oxidase, urease and casein hydrolysis. Identification of actinomycetes genera and species based on the morphology and biochemical properties indicated potential strain of marine actinomycetes^[20].

Out of 73 hospital isolates, 48 showed Gram-positive types of infection and 25 found to be Gram-negative. Totally 48 isolates were recorded and identified as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, *P. vulgaris*, *Enterococcus* species and *S. aureus*. The highest number of patients with hospital infections was of the age range of 40-50 y followed by the age range 50-60 y. The frequency of pathogens in our study was *E. coli* (35.41%) and *K. pneumoniae* (16.66%), *P. aeruginosa* (12.5%), *P. mirabilis* (10.41%), *P. vulgaris* (6.25%), *Enterococcus* species (10.41%) and *S. aureus* (8.33%) as shown in fig. 1. The most common hospital-acquired infection was caused by *E. coli*, the predominant bacteria found in hospitalized patients^[21]. All tested pathogens were analysed by MAR index using standard antibiotics. The antimicrobial sensitivity of isolated clinical pathogens revealed that 65.6% of isolates were found to be MDR. Among the isolates tested, *E. coli* was found in 0.687, *K. pneumoniae* as 0.625 and lowest MAR index found in Gram-positive isolates *Enterococcus* species and *S. aureus* as 0.441, respectively (fig. 2). The highest multiple antibiotic resistant indices (MARI) for *E. coli* were 0.687. The overall rate of resistance against *E. coli* was worldwide reported, which was similar with this

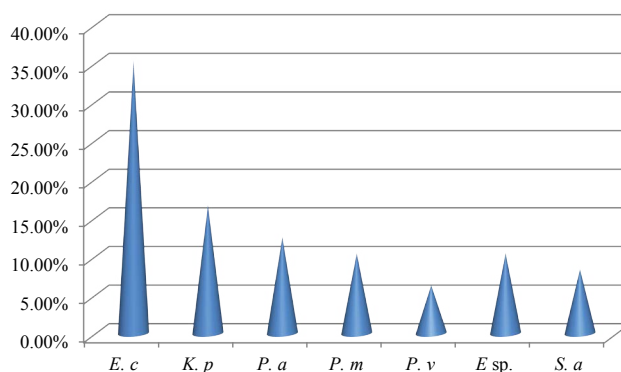


Fig. 1: Distribution of bacterial isolates
E. c: *Escherichia coli*; *K. p:* *Klebsiella pneumoniae*; *P. a:* *Pseudomonas aeruginosa*; *P. m:* *Proteus mirabilis*; *P. v:* *Proteus vulgaris*; *E sp.:* *Enterococcus* species; *S. a:* *Staphylococcus aureus*

study^[22]. MAR index is a tool that reveals the spread of bacteria resistance in a given population^[23]. MAR index greater than 0.20 implies that the strains of such bacteria originate from an environment where several antibiotics are used. The MAR indices obtained in this study is a possible indication that a very large proportion of the bacterial isolates have been exposed to several antibiotics. These bacteria are common environmental organisms, which act as opportunistic pathogen in clinical cases where the defence system of the patient was compromised to broad spectrum antibiotics particularly to penicillin and cephalosporins^[24].

About 32 broad spectrum antibiotics were tested against hospital pathogens. Antibiotic sensitivity revealed that the high degree of resistance (50-100%) was reported against cephalosporin, monobactam and ampicillin. Ampicillin resistance of 100% was significantly reduced to 28% by clavulnate. Significant resistant ($\leq 50\%$) was observed against fluoroquinolones derivatives. Among the tested antibiotics, 7 antibiotics belonged to amikacin, carbapenems, tazobactam and cepahatoxime clavulnate showed potent antibacterial activity against tested pathogens. No resistance was observed against these antibiotics. It has been reported that meropenem is the most effective antibiotic against Gram-negative and Gram-positive uropathogens in the rate of 93-100%^[25].

Out of 34 antibiotics tested against Gram-positive *S. aureus* and *Enterococcus* species, 100% of resistance was developed against penicillin and methicillin (fig. 3). *S. aureus* showed 75% resistance against doxycycline and no resistance against vancomycin. *Enterococcus* species showed 60% resistance against vancomycin and no resistance against doxycycline. As described previously, methicillin resistance was associated with resistant to other antibiotics^[26]. In the present study, the fact that high prevalence of MRSA infections have showed sensitivity to vancomycin encourages the usage of vancomycin rather than doxycycline^[27].

The antimicrobial activity of *Micromonospora* species KPMS10 was given in Table 1. Potent antibacterial effect was recorded as 18 mm against *K. pneumoniae* and *E. coli*, moderately active against *P. aeruginosa* (15 mm), *P. vulgaris* (16 mm), *Enterococcus* species

(16 mm) *S. aureus* (14 mm) but least activity against *P. mirabilis* (13 mm). It was found that meropenem was more effective than actinomycetes. *Micromonospora* species are the common inhabitants that have proven to be a constant source of novel bioactive compounds with structural and functional diversity giving rise to antibacterial, antifungal, antiviral, anticancer activity^[28]. Marine actinomycetes has the unique distinction that various secondary metabolites can be produced by a single strain^[29].

In this study, AgNPs were biosynthesized with the help of culture filtrate of *Micromonospora* species KPMS10. It showed pale yellow colour before the addition of silver ions, which changed to brownish colour. No colour change was observed without the addition

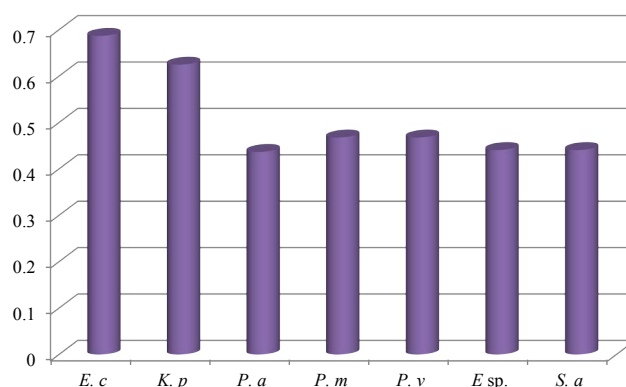


Fig. 2: Multiple antibiotic resistance (MAR) index

E. c: *Escherichia coli*; *K. p*: *Klebsiella pneumoniae*; *P. a*: *Pseudomonas aeruginosa*; *P. m*: *Proteus mirabilis*; *P. v*: *Proteus vulgaris*; *E sp.*: *Enterococcus species*; *S. a*: *Staphylococcus aureus*

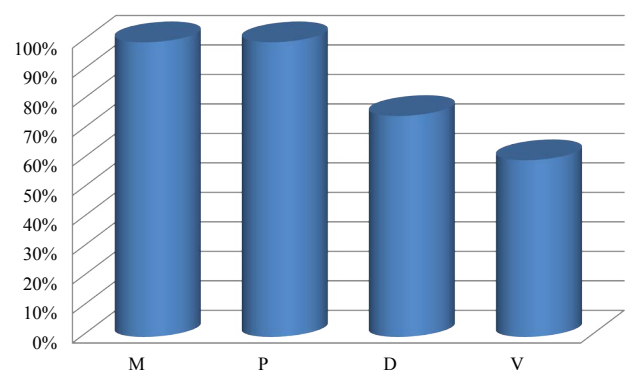


Fig. 3: Percent of Gram-positive resistant isolates against tested antibiotics

M: methicillin; P: penicillin; D: doxycycline; V: vancomycin

TABLE 1: ANTIBACTERIAL ACTIVITY OF MARINE ACTINOMYCETES AGAINST MULTIDRUG-RESISTANT PATHOGENS

Strain code	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>	<i>S. aureus</i>	<i>Enterococcus sp.</i>
KPMS10	18±0.04	18±0.036	15±0.02	16±0.04	13±0.4	14±0.04	16±0.02
Meropenem	18±0.02	18±0.02	18±0.1	18±0.04	20±0.1	20±0.2	20±0.04

Zone of inhibition mm in diameter, mean±SD, n=3. Among the six tested strains, KPMS10 showed potent antibacterial activity

of silver nitrate in culture supernatant. Surprisingly, the intensity of brown colour increased periodically and was maintained throughout the experiments. The appearance of a pale yellow colour to brownish colour in the solution containing biomass became an indicator of the formation of AgNPs^[30].

Biosynthesized AgNPs were further confirmed using UV/Vis spectrophotometer. A strong and broad plasmon absorption peak was observed at 450 nm in UV/Vis surface (fig. 4). This might occur due to the reduction of metal ions by secondary metabolites present in the cell. The absorption peaks indicated the presence of biosynthesized AgNPs. Homogenous AgNPs have been known to produce a surface plasmon resonance band at the range of 420-450 nm^[31]. An absorption peak at 450 nm by *S. glaucus* was previously reported^[32].

FTIR measurements were carried out to identify possible interaction between silver and protein molecules, which could be responsible for synthesis, stabilization of well dispersed AgNPs in the reaction mixture^[33]. FTIR revealed a spectrum at 3465.36 and 3434.17 cm⁻¹, which is assigned to the hydrogen bonded OH stretch, peak as 2074.39 cm⁻¹ corresponding to carboxylic acid group (fig. 5a). Similarly, peak 1635.77 is assigned to the primary or secondary amines (N-H). The FTIR studies confirmed the fact that COOH derivative

have strong ability to bind silver and to stabilize the synthesis of nanoparticles^[34].

Smaller size nanoparticles effectively penetrated to cell due to its larger surface availability for interaction and interfere with metabolism of cell. In this study, SEM analysis shows that individual silver particles as well as a number of aggregates. The SEM analysis reveals that size of nanoparticles was found to be 80 nm (fig. 5b). The biomolecules present in the surface of nanoparticles leads to agglomeration structure. Similar results were observed by *Bacillus licheniformis* mediated AgNPs^[35].

The present study showed antibacterial activity of silver ions against all tested pathogens. The maximum zone of inhibition of 24 mm was found with *E. coli* and *K. pneumoniae* with 75% of relative zone of inhibition (RIZD). About 22 mm zone of inhibition was observed against *P. aeruginosa* and *Enterococcus* species with 55 and 66% RIZD (Table 2). These results suggested that the AgNPs produced were more potent than meropenem. Antimicrobial activity was reported to be due to the penetration of AgNPs into the drug resistant bacteria resulting in disruption of cell membrane and release of cell contents^[36]. Antimicrobial property of AgNPs depended on the size of nanoparticles^[37].

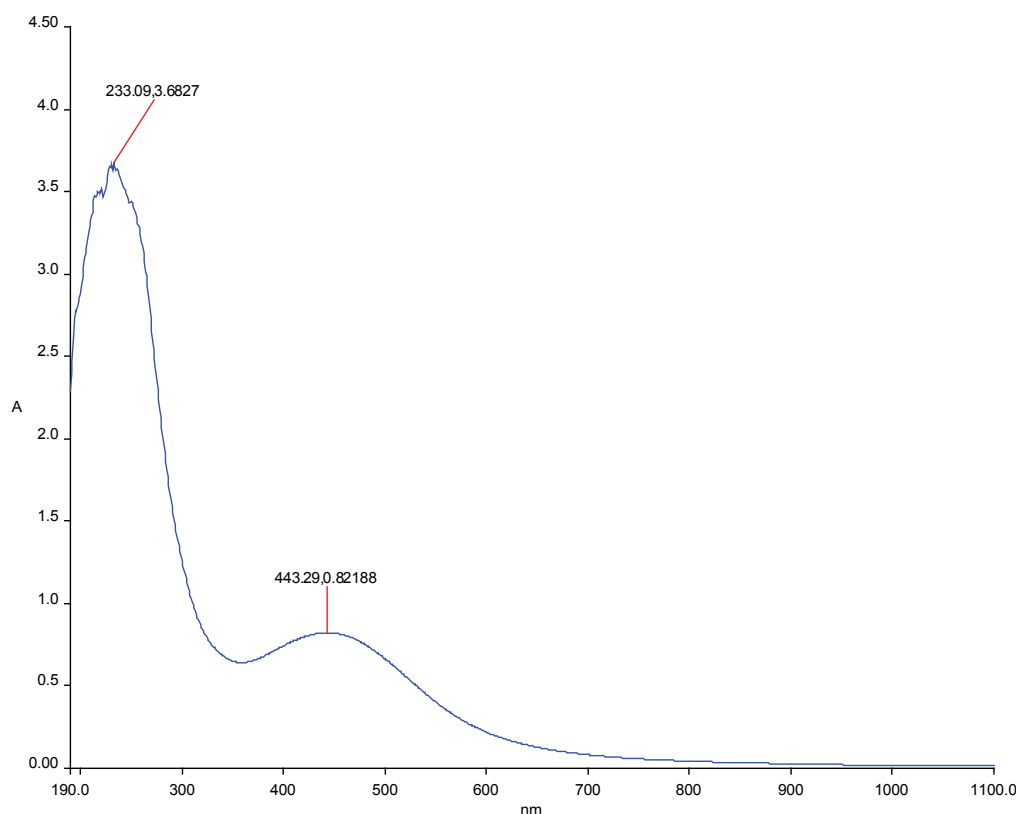
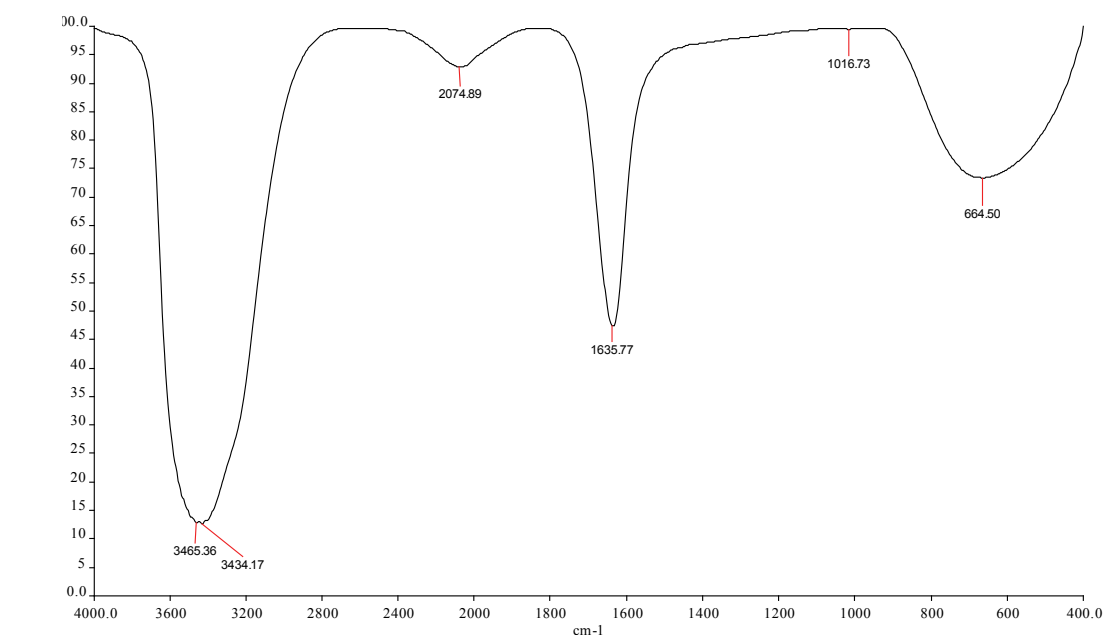
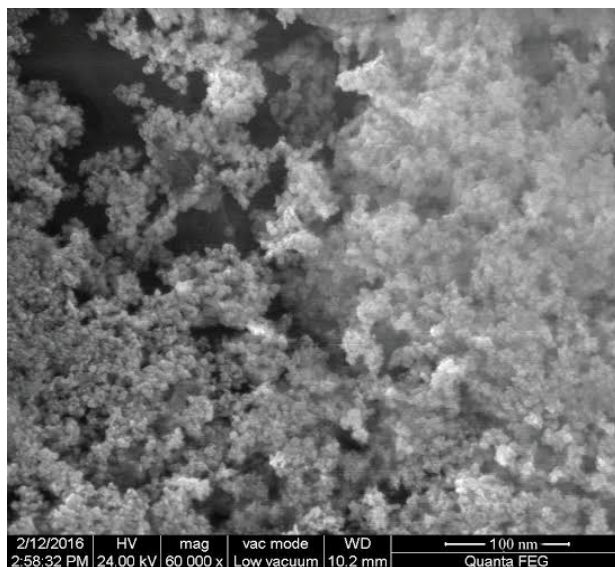


Fig. 4: Characterization of AgNPs using UV/Vis spectroscopy



a.



b.

Fig. 5: Characterization of AgNPs using FTIR and SEM

a. Characterization of AgNPs using FTIR analysis; b. SEM image of *Micromonospora* sp. KPMS 10**TABLE 2: ANTIMICROBIAL ACTIVITY OF BIOSYNTHESIZED AgNPs AGAINST MULTIDRUG-RESISTANT PATHOGENS**

Components	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>	<i>S. aureus</i>	<i>Enterococcus</i> sp.
AgNPs B	24	24	20	22	20	18	22
Cephalexime	16	16	16	18	16	16	18
Negative control	12	12	12	12	12	12	10
RIZD (%)	75	75	44	55	44	37	66

Zone of inhibition mm in diameter

It was found that smaller AgNPs synthesized by microorganisms had a greater antibacterial activity.

The AgNPs showed no haemolytic activity at 10 and 20 µg/ml, slight haemolysis at 30 µg and significant haemolytic activity at 50 µg/ml after 12 h incubation.

Ninety percent haemolysis was observed at 50 µg/ml after 12 h incubation. The lower haemolytic activity observed at 25 µg could lead to adverse effects. Based on the results, the safe range of AgNPs was 10 to 20 µg/ml. Negative control (Tyrode) and positive control

(Triton X-100 1%) induced 0 and 100% haemolysis, respectively (fig. 6). The determination of haemolysis is based on haemoglobin absorbance at 550 nm, with subtraction of the interference of AgNPs. Various studies have reported haemolytic activity of AgNPs, and our results were in agreement with those reported in previous studies about the prohaemolytic properties of AgNPs^[38].

Phylogenetic analysis (fig. 7) of 16S rRNA sequence analysis showed that the *Micromonospora* species KPMS10 was closely related to *Micromonospora coxensis* (98%) and the Genbank accession number was KU867645. Currently, taxon actinomycetes are accommodates spore forming Gram-positive bacteria that from extensive branching substrates on aerial mycelia. Based on these studies, the actinomycetes strains were recorded^[39].

In conclusion, application of isolated marine *Micromonospora* species in nanotechnology could meet the challenges in pharmaceutical industry for developing new antibacterial compounds to treat existing infectious diseases. Marine derivative could provide a promising source of structurally diverse secondary metabolites from actinomycetes with potential activity against multidrug-resistant pathogens. Further studies are needed to identify the active lead molecule.

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Conflicts of interest:

There are no conflicts of interest.

Financial support and sponsorship:

Nil.

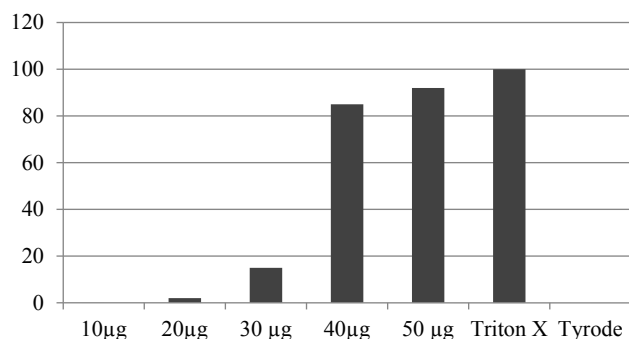


Fig. 6: Haemolytic effect of synthesized AgNPs

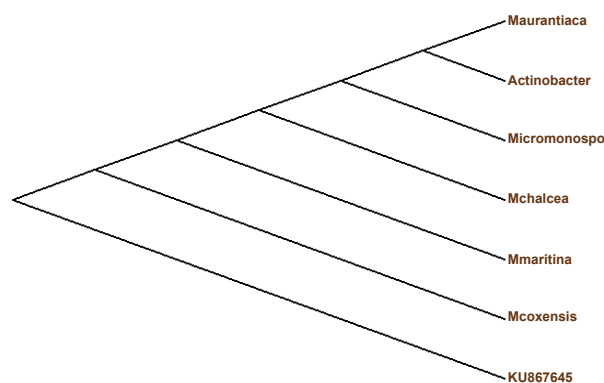


Fig. 7: Phylogenetics analysis of *Micromonospora* species KPMS10

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