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Antibacterial potential of silver nanoparticle synthesized by marine actinomycetes in reference with standard antibiotics against hospital acquired infectious pathogens

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Multi resistance to antibiotics is a serious and disseminated clinical problem, common to several new compounds that block the resistance mechanism. The present study aimed at the comparative study of silver nanoparticles synthesized through actinomycetes and their antimicrobial metabolites with standard antibiotic. Marine actinomycetes collected from Gulf mannar costal region, Kayalpatnam, located at Tuticorin district, Tamil nadu, India. Totally, five actinomycetes were isolated and identified based on their spore formation and biochemical studies. Three isolates belonged to the genera of *Streptomyces* sp and two were M*icromonospora sp*. *Streptomyces* sp KPMS3 showed potent antibacterial activity against Gram negative pathogens. Gram positive isolates are sensitive to *Micromonospora* sp (KPMM2). Among the five isolates, isolate *Micromonospora* sp (KPMM2) was found to be an effective silver nanoparticle synthesizer. The obtained silver nanoparticles were characterized using UV-Vis spectroscopy, FTIR and TEM. The morphology of nanoparticle is found to be spherical and an average size of ranges between 38 to 52 nm. The antimicrobial activities of silver ion against test pathogens were found to be superior to cephalosporin antibiotic. The *in vitro* hemolytic assessment of silver nanoparticles were found to be non-hemolytic at maximum of 20 µg/ml. It was found that smaller silver nanoparticles synthesized by microbial route had a greater antibacterial activity and less hemolytic in nature.

Key words: ESBL, cefotaxime, drug resistant and hemolysis.

INTRODUCTION

Hospital acquired infection are a cause of prolonged hospital stay and contagious which may lead to high morbidity and mortality throughout the hospital patients. Hospital acquired infection constitute an economic

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worries on health care. It is estimated that 80% of all hospitals death is directly or indirectly linked to Hospital acquired infection (Hughes et al., 2005). In India, 10 to 30% patients are admitted in hospitals or nursing homes are associated with hospital acquired infection. The failure of this infection due to the development of drug resistance organisms and the rate is 3.4% (Khoiee et al., 2008). Antibiotic resistant hospital infection can be especially deadly because antibiotics are used intensely in hospitals compared with the community and frequent use drives the development of multi drug resistance bacteria. Microbes are known to fit on inanimate source such as touch surface for extending period of time. This can be making troublesome in hospital environments where patients with weakened immunity are at increased risk for contracting hospital acquired infection (Hidron et al., 2008). The spreading of drug resistant bacteria is stopped by using the application of nanotechnology in order to synthesis nanoparticle. Nanoparticles (Nps) are focused as fundamental building blocks of nanotechnology, particularly silver nano particles. Indeed over a past several years, silver nano particle are synthesized by a potential new antibiotics developer actinomycetes from marine sediments (Absar et al., 2003).

Actinomycetes are free living gram positive saprophytic bacteria and it has a major source for production of numerous natural biological metabolites especially antibiotics. Silver nanoparticle play an important role in many field such as nano crystalline silver dressings, creams, gel effectively reduce bacterial infections in chronic wounds (Ip et al., 2006) and also used to control bacterial infectious disease (Smriti et al., 2012). Silver is the metal of interest and it provides a most durable antimicrobial protection against microorganism. Nano sized silver particle is the most promising source to kill microbes very effectively because that act on both intra and extracellularly. Silver nanoparticle exhibit as a strong bactericidal agent and depicts activity against both multi drug resistant gram positive and negative bacteria (Zeng et al., 2007; Roe et al., 2008). Marine actinomycetes will develop a valuable resource for novel metabolites of pharmaceutical and medicinal interest, including antimicrobial agents (Mitsuiki et al., 2002). The objective of this work was to synthesize characterize and evaluate the efficacy of biosynthesized Ag Nps against nosocomial pathogens.

MATERIALS AND METHODS

Sample collection

Systematic screening of actinomycetes from marine sediments was done by random sample collection between 200 m distance at a depth of 5 m using core sampler from Gulf mannar costal region, Kayalpatnam, located at Tuticorin district, Tamil nadu, India. The central portion of the marine sediments were aseptically transferred to the sterile bottles during June 2013 and brought up to laboratory with help of ice bag. The sediments sample was blackish brown colour and of a sandy texture.

Isolation of actinomycetes

All the marine sediments were air dried to minimize bacterial contaminants. One gram sediments were serially diluted up to 10^{-6} dilution. One ml of diluted sample was permitted in to the Petri plate followed by Starch casein nitrate agar (SCNA) medium supplemented with cyclohexamide 50 µg/ml and nystatin 50 µg/ml. After solidification, all the plates were incubated at 28°C for 7 to 15 days until the colonies were developed.

Spore morphology

All the isolates were identified by slide culture method. A drop of 0.1% tryphan 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.

Antibacterial activity of secondary metabolites

Antimicrobial activity of actinomycetes culture filtrate was analyzed with agar well diffusion method. The 24 h nutrient broth culture of multidrug resistant pathogens such as ESBL *Escherichia coli*, ESBL *Klebsiella pneumonia, Enterococcus faecium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Proteus mirablis* and *Proteus vulgaris* were isolated from hospital acquired infection and bio assayed against standard antibiotics. 100 µl of actinomycetes culture filtrate were used as a test sample against clinical isolates. Inoculated plates were incubated at 37°C for 24 h. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

Bio synthesis and characterization of silver nanoparticles

The isolated actinomycetes culture was inoculated in to ISP IV medium and incubated at 28°C for 7-15 days. Actinomycetes broth medium was centrifuged at 5000 rpm for 10 to 20 min to remove cell debris. 10 ml of actinomycetes cultured filtrate was permitted in to the Ag NO_3 aqueous solution and incubated at room temperature in a shaker under dark condition. Bio reduction was monitored by colour change and UV spectrum analysis. Further the nanoparticles were collected at 15, 000 rpm centrifugation and characterized by FTIR and SEM analysis.

Antibacterial activity of silver nanoparticles

Antimicrobial activity of biosynthesized nano particle was analyzed with agar well diffusion method. The multidrug resistant pathogen such as ESBL *E. coli*, ESBL *K. pneumonia, E. faecium*, *S. aureus*, *P. aeruginosa, P. mirablis* and *P. vulgaris* were isolated from hospital acquired infection and inoculated into nutrient broth and incubated at 37°C for 24 h. After incubation, the test pathogens were inoculated on Muller Hinton agar (MHA) by using sterilized cotton swabs. In each of these plates, wells were cut out using a sterilized gel borer and 50 µl of biosynthesized Ag NPs were used as a test sample against clinical isolates. Inoculated plates were incubated at 37°C for 24 h. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

Haemolysis assay of Ag NPs

The cytotoxic effect of silver nanoparticles was studied by

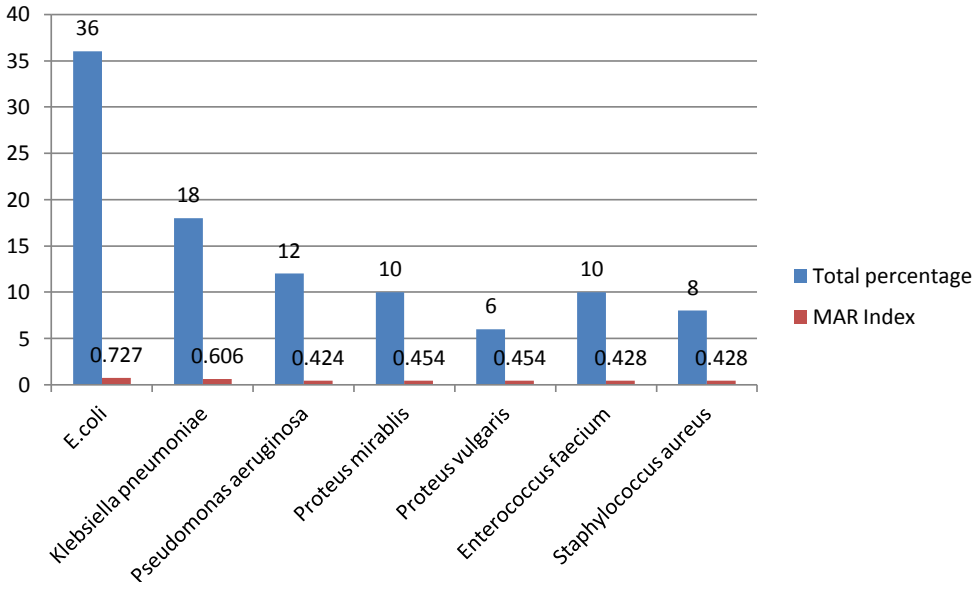

Figure 1. Distribution of isolated pathogen and its MAR index.

performing hemolytic test (Krajewski et al., 2013).Two milliliters (2 ml) of blood was mixed with 0.2 M PBS (pH 7.0) and centrifuged at 10000 rpm for 10 min. The pellet of RBC was collected and washed three times by PBS at 10000 rpm for 3 min. The obtained RBC was diluted with PBS at 1:10 ratio. 10 µL of nanoparticles in tyrode (1, 5, 10, 25 and 50 µg/ml), tyrode (negative control) and triton X-100 (positive control) were added to 290 µL of washed RBC. The suspension was incubated at room temperature by shaking plate for 12 h. After incubation, the suspension was centrifuged at 10000 rpm for 5 min. Supernatant was recorded in spectrophotometer at 550 nm. The haemolysis (H) was calculated as:

H (%) = (OD sample – OD tyrode) / (OD Triton X-100 1% - OD tyrode)*100.

RESULTS AND DISCUSSION

Isolation of marine actinomycetes

The present work was used to carry out the synthesis of silver nanoparticle by marine actionomycets collected from Gulf Mannar Costal Region, Kayalpatnam, located at Tuticorin district, Tamil nadu, India. Based on the cultural characterization of Actinomycetes were identified as *Streptomyces* sp(KPMS) and *Micromonospora* sp (KPMM). All the isolated Actinomycetes strains were Gram positive but differ morphologically by producing different mycelium and spore. *Streptomyces* sp showed white coloured aerial mycelium and dark brown substrate mycelium with refractile spiral spore. 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* sp (Kawamoto, 1989). Currently, taxon actinomycetes are accommodates spore forming gram positive bacteria that from extensive branching substrates and aerial mycelia. Based on this developments, the actinomycetes strains were recorded (Waksman, 1961).

Isolation of drug resistant pathogens

Out of 34 samples, 28 showed poly microbial infection and six were found to be negative. Totally, 50 isolates were recorded and identified as *E. coli, K. pneumoniae, P. aeruginosa, P. mirablis, P. vulgaris, E. faecium* and *S. aureus.* The highest numbers of patients with nosocomials were found within the age range of 40 to 50 followed by the age range 50 to 60. Figure 1 represents the frequency and MAR index of tested isolates against standard antibiotics. The percentages of frequency of isolated pathogens were 36 ≥ 18 ≥ 12 ≥ 10 ≥ 06 ≥ 10 ≥ 8. The most common nosocomial pathogens in our study were *E. coli* (36%) and *K. pneumonia* (18%). *E. coli* was the predominant bacteria found in hospitalized patients (Manikandan et al., 2011). The antimicrobial sensitivity of isolated clinical pathogens reveals that 76 percent of isolates were found to be MDR. Among the tested genera's *E. coli* and *K. pneumonia* showed high degree of resistance against all tested antibiotics. The MAR indices of indigenous bacterial isolates ranges was found in 0.727 to 0.424 (Figure 1). The highest multiple antibiotic resistant indices (MARI) for *E. coli* were 0.727. Mandal et al. (2001) determined that the overall rate of resistance against *E. coli* was worldwide reported which was similar with this study. Multiple antibiotics resistance (MAR) index is a tool that reveals the spread of bacteria resistance in a given population (Krumpermann, 1983). A

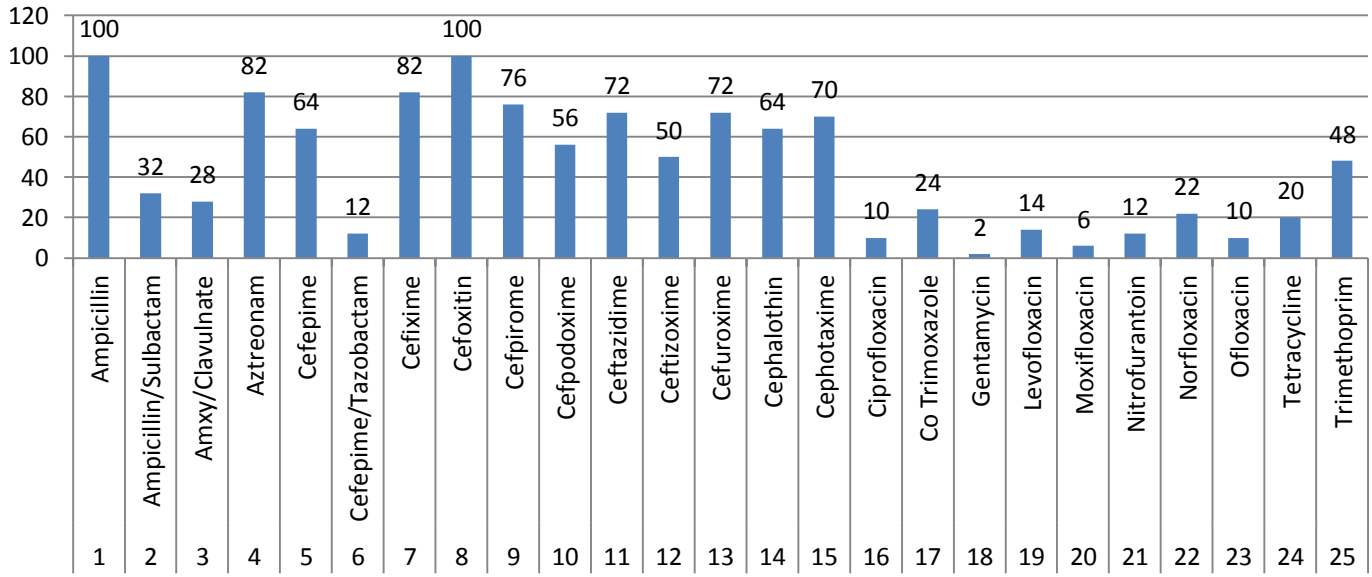

Figure 2. Percentage of Gram negative Resistant Isolates against tested Antibiotics.

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 bacteria isolates have been exposed to several antibiotics. These bacteria are common environmental organisms which act as opportunistic pathogen in clinical cases were the defense system of the patient is compromised to broad spectrum antibiotic resistance particularly penicillin and cephalosporins (Obritsch et al., 2004).

Out of 32 broad spectrum tested antibiotics, 13 antibiotics were come under cephalosporin groups, two were aminoglycosides (amikacin and gentamycin), five antibiotics come under fluoroquinolones (ciprofloxacin, ofloxacin, norfloxacin, moxifloxacin and levofloxacin), four penicillin derivatives (ampicillin, ampicillin/sulbactam, amoxy/clavulnate and piper tazobactam), two of sulfonamides (trimethoprim and cotrimoxazole), three of carbapenems and one of tetracycline, nitrofurans and monobactam (aztreonam) were tested against 50 isolates. Antibiotic sensitivity reveals that the high degree of resistance (50 to 100%) was reported against cephalosporin, monobactam and ampicillin. The 100% of ampicillin resistance were significantly reduced into 28% by clavalunate (Figure 2). Significant resistance (≤ 50%) was observed against fluoro quinolones derivatives. Among the tested antibiotics seven of antibiotics belong to amikacin, carbapenems, tazobactam and cepahotoxime clavulnate showed potent antibacterial activity against tested pathogens. No resistance was observed against these antibiotics. It has been reported that amikacin is the most effective antibiotic against *E. coli* and *K. pneumonia* (Schaeffer et al., 2001). Our result was further supported by another study where the susceptibility rate of *E. coli* to amikacin remained 93 to 100%. Out of four tested Gram positive antibiotics against *S. aureus* and *E. faecium* 100% of resistance was isolated against penicillin and methicillin (Figure 3). *S. aureus* showed 33% of resistance against doxycycline and no resistance was found against vancomycin. *E. faecium* showed 33% of resistance against vancomycin and no resistance was observed against doxycycline. As described previously, methicillin resistance was associated with resistance to other antibiotics (David and Daum, 2010). In the present study, high prevalence of MRSA infection have showed sensitive to vancomycin encourages the usage of vancomycin than doxycycline (Appelbaum, 2007).

Antibacterial activity of marine actinomycetes isolates

The antimicrobial activity of culture filtrate reveals that *Streptomyces* sp KPMS 3 showed potent antibacterial effect (18 mm) against ESBL *K. pneumoniae* and *E. coli,* moderately active against *P. aeruginosa and P. vulgaris* but less significantly active against *P. mirablis. S. aureus* and *E. faecium* were not sensitive against *Streptomyces* sp but highly sensitive against *Micromonospora* sp. Of these two isolates of *Micromonospora* sp KPMM1 showed antibacterial activity against *S. aureus* and *E. faecium* (Table 1). It was found that amikacin was more effective than actinomycetes due to the productivity and purity of metabolites during fermentation. *Micromonospora* and *Streptomyces* sp the common inhabitants and have proved to be a continuing source of

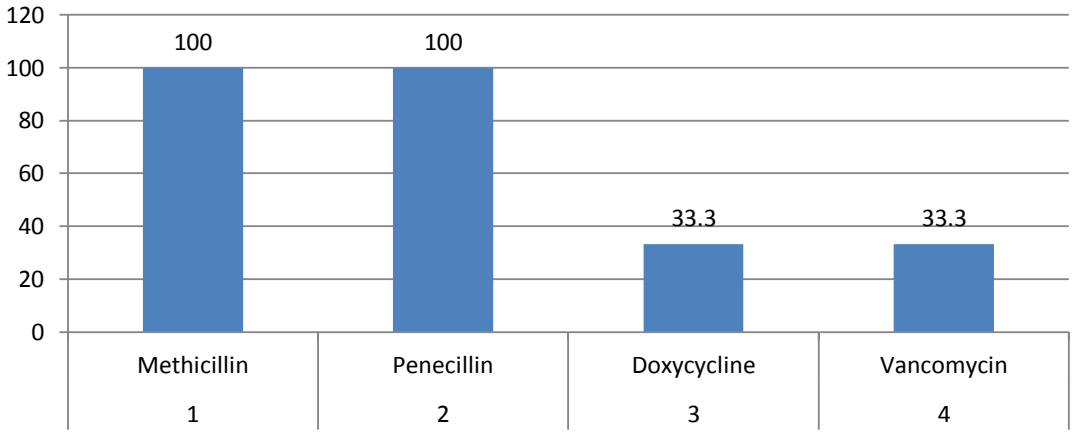

Figure 3. Percentage of gram positive resistant isolates against tested antibiotics.

Table 1. Antibacterial activity of Marine Actinomycetes against Multi drug resistant pathogens.

Strain code	*ESBL E coli*	*ESBL Klebsiella pneumoniae*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Proteus mirablis*	*Staphylococcus aureus*	*Enterococcus faecium*
KPMS 1	-	-	-	-	-	-	-
KPMS 2	-	-	-	-	-	-	-
KPMS3	18±0.04	18±0.036	15±0.02	16±0.04	12±0.4	-	-
KPMM1	-	-	-	-	-	14±0.04	16±0.02
KPMM2	-	-	-	-	-	-	-
Amikacin	18±0.02	18±0.02	18±0.1	18±0.04	20±0.1	20±0.2	20±0.04

novel bioactive compound having great structural and functional diversity including antibacterial, antifungal, antiviral, anticancer agents (Koehn and Carter, 2005). Actinomycetes that produce secondary metabolites often have the potential to produce various compounds from a single strain (Schiewe and Zeeck, 1999).

Bioreduction of silver nitrate

The culture filtrate of *Micromonospora* sp showed pale yellow color before the addition of silver ions and converted to brownish colour. The appearance of pale yellow color to brownish color in the solution containing biomass was indication of the formation of silver nanoparticles (Ag NPs) (Sastry et al., 2003) further confirmed at 450 nm UV- Visible surface Plasmon absorption peak (Figure 4). The homogenous Ag Nps are known to produce the surface Plasmon resonance band at the range of 420 to 450 nm (Duran et al., 2007). The absorption peak at 450 nm by *Streptomyces glaucus* was observed by one another researcher (Tsibakhashvili et al., 2011). FT-IR measurements was carried out to identify possible interaction between silver and protein molecules which may be responsible for synthesis, stabilization and well dispersed silver nanoparticles in the reaction mixture (Navin et al., 2011). FT-IR revealed a spectrum at 3467. 19 and 3435. 06 cm^{-1} which is assigned to the hydrogen bonded OH stretch, peak such 2386 cm-1 corresponding to carboxylic acid group. Similarly, peak 1635 is assigned to the primary or secondary amines (N-H) (Figure 5). The FT-IR studies confirmed the fact that COOH derivative have strong ability to bind silver and to stabilize the synthesis of nanoparticles (Mervat et al., 2012).

Antibacterial activity of biosynthesized silver nanoparticles

Antibacterial study of silver ions showed broad spectrum antibacterial activity against all tested pathogens. The maximum zone of inhibition was 24 mm against ESBL *E. coli* and ESBL *K. pneumoniae* with 75% of relative zone of inhibition (RIZD*).* 22 mm zone of inhibition was recorded against *P. aeruginosa* and *E. faecium* with 55 and 66% RIZD (Table 2). These result suggested that the biosynthesized silver nanoparticles are highly potent than amikacin and cephalosporin antibiotic. The antimicrobial activity was reported to be due to the penetration of Ag

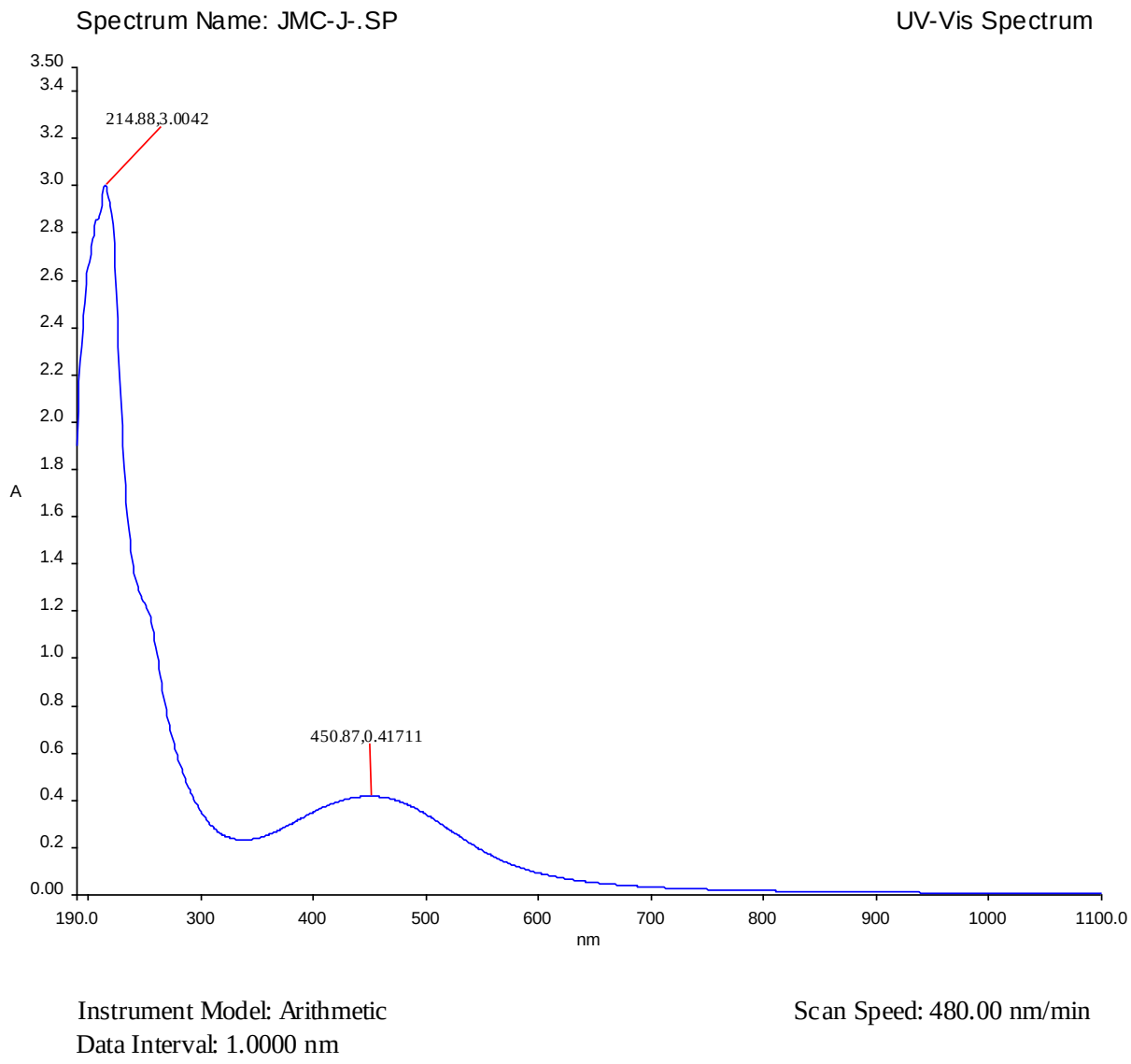

Figure 4. Characterization of silver synthesized nanoparticle by U-vis spectroscopy.

Nps into the drug resistant bacteria, damaging the cell membrane and released cell contents (Panacek et al., 2006). Antimicrobial property of silver nanoparticles depends on the size of nanoprticles synthesized (Richard et al., 2012). Smaller size of nanoparticle effectively penetrates to cell due to its larger surface availability for interaction and interfere the metabolism of cell. In this study, the TEM analysis reveals that (Figure 6) size of nanoparticles was found to be 38 and 52 nm, respectively. The biomolecules present in the surface of nanoparticles leads to agglomeration structure. The similar results were observed by *Bacillus licheniformis* mediated silver nanoparticles (Kalimuthu et al., 2008). The application of silver in combination with microbial system would be effective in enhancing its antimicrobial activity.

Hemolytic effect of silver nanoparticles

Figure 7 shows the synthesized nanoparticles showed no hemolytic activity at 10 and 20 µg/ ml but less significantly at 30 µg and have significant hemolytic activity at 50 µg/ml after 12 h incubation. 90% Haemolysis was observed at 50 µg/ml after 12 h incubation. A lower hemolytic activity was observed at 25 µg can lead to adverse health effects. Result showed that the concentration of Ag Nps is safe at the range of 10 to 20 µg/ml. Negative control (tyrode) and positive control (triton X-100 1%) induced 0 and 100%, respectively of hemolysis. The determination of hemolysis is based on hemoglobin absorbance at 550 nm, with subtraction of the interference of Ag NPs. Although, various studies evaluated the hemolytic activity of Ag NPs, our results

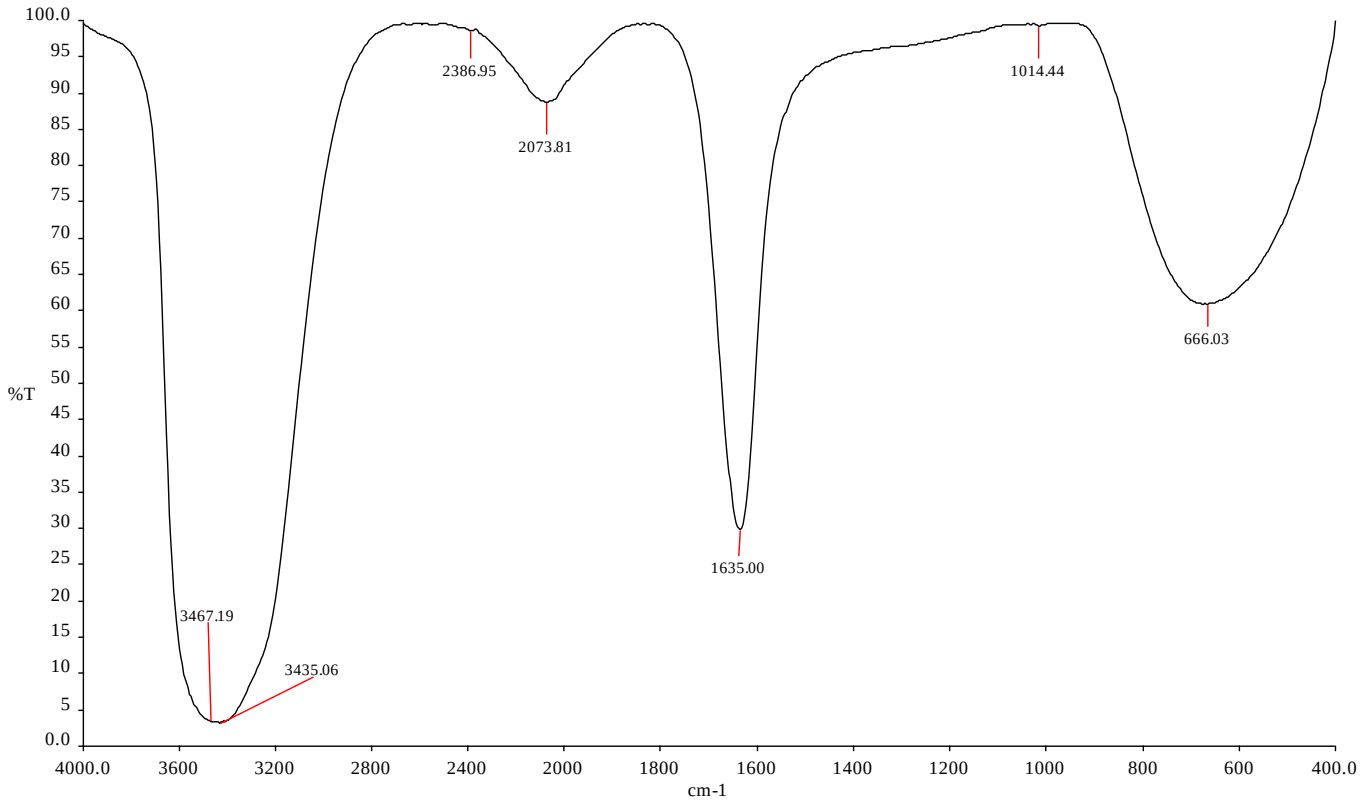

Figure 5. Characterization of silver synthesized nanoparticle by FTIR analysis.

Table 2. Antimicrobial activity of biosynthesized silver nanoparticles against MDR pathogens.

Sample	*ESBL E. coli*	*ESBL K. pneumoniae*	*P. vulgaris*	*P. aeruginosa*	*P. mirablis*	*S. aureus*	*E. faecium*
AgNP B	24	24	20	22	20	18	22
Cephotaxime	16	16	16	18	16	16	18
Negative control	12	12	12	12	12	12	10
RIZD	75	75	44	55	44	37	66

are in agreement with previous studies that concluded to the prohemolytic properties of Ag NPs (Choi et al., 2011). These results may have a clinical impact at high concentration of Ag NPs, since a release of hemoglobin can lead to adverse health effects such as anaemia.

Conclusion

Many of these secondary metabolites are potent antibiotics, which have been made by marine *Streptomyces* sp, the primary antibiotic-producing organisms exploited by the pharmaceutical industry. It is suggestive that the exploitation of *Streptomyces* sp. in nanotechnology has recently received considerable attention. Marine source provides a promising source of Actinomycetes that can ruin the multidrug resistant pathogens. Silver nanoparticles have an important advantage over conventional antibiotics in that they kill all pathogenic microorganisms, no organisms has ever been reported to readily develop resistance to it because of their high reactivity that is due to the large surface to volume ratio. The synthesis of nanomaterials of specific composition and size is a burgeoning area of materials science.

Conflict of interest

The authors have not declared any conflict of interest.

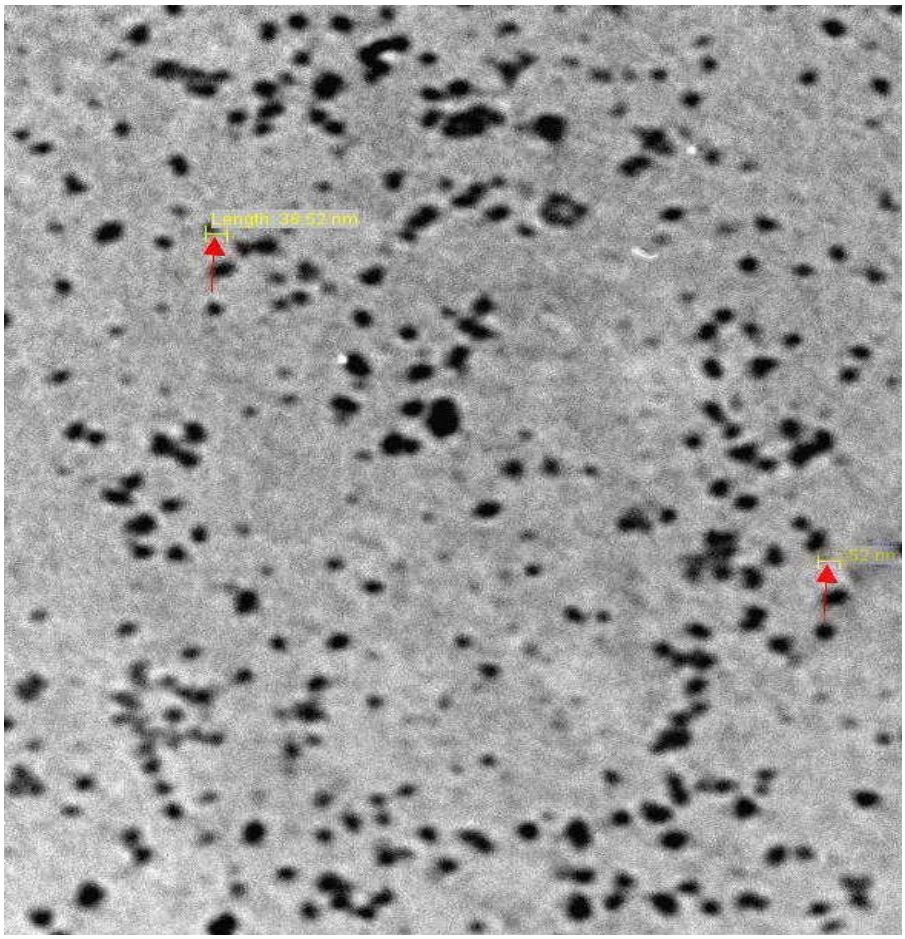

Figure 6. TEM image of microbial and chemically synthesized silver nanoparticle.

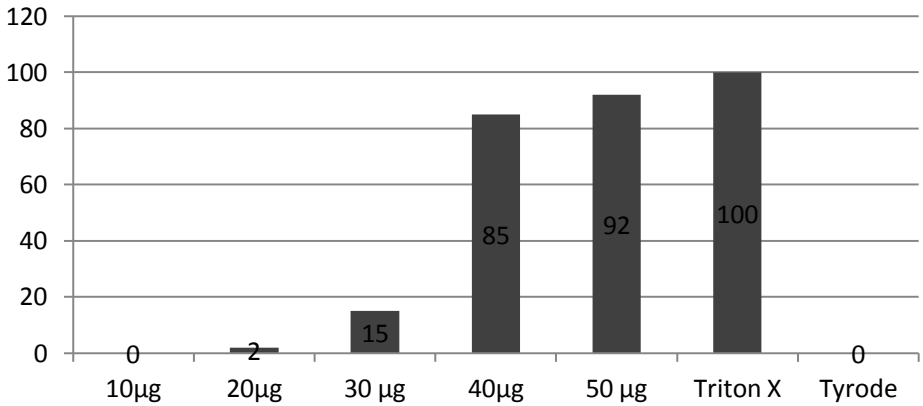

Figure 7. Hemolytic effect of synthesized silver nanoparticles.

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