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BIOSYNTHESIS OF SILVER NANOPARTICLES USING *CALOTHRIX MEMBRANACEA* KLR006 AND CHARACTERIZATION OF ITS ANTIMICROBIAL PROPERTIES

Veerasamy Pushparaj Ramya*, Gangatharan Muralitharan

Department of Microbiology, Centre of Excellence in Life Sciences, Bharathidasan University, Palkalaiperur, Tiruchirappalli, Tamilnadu, India.

ABSTRACT: Silver nanoparticles (AgNPs) synthesizedfrom freshwater strain of *Calothrix membranacea* KLR006was characterized for chemical, physical and biological properties. An aqueous solution of silver ions was treated with a live biomass of *Calothrix membranacea* KLR006for the formation of AgNPs. These nanoparticles showed an absorption peak at 300- 700 nm in the UV-visible spectrum and scanning electron microscope (SEM)analysis showed that the nanoparticles were embedded within an organic matrix. Fourier transform-infrared spectroscopy (FT-IR) analysis confirmed the presence of phenolic compounds in the aqueous extracts of *Calothrix membranacea*KLR006 and acted as reducing and capping agents. X- ray diffraction (XRD) analysis was also carried out to demonstrate the crystalline nature of the biosynthesized AgNPs.The antimicrobial activity results determined by an agar well diffusion method demonstrated a significant antibacterial activity of the biosynthesized silver nanoparticles against pathogenic microbes tested, even towards the drug resistant bacterial pathogens.

KEYWORDS: *Calothrix membranacea*, silver nanoparticles, biosynthesis, SEM, EDAX, FT-IR, XRD, antibacterial activity.

Corresponding Author: Dr. Veerasamy Pushparaj Ramya* Ph.D.

Department of Microbiology, Centre of Excellence in Life Sciences, Bharathidasan University, Palkalaiperur, Tiruchirappalli, Tamilnadu, India.

1.INTRODUCTION

Nanoscience is an emerging subject that utilizes the fundamental properties of nanosized objects [1, 2]. The optical, electronic, magnetic, and catalytic properties of nanoparticles are unique than the

bulk materialsdue to their high surface area to volume ratio [3, 4]. Silver and gold metal nanoparticles show different colors dueto their Surface Plasmon Resonance (SPR) phenomenon. It is a collective oscillation of free electrons of the metalnanoparticles in resonance with the frequency of the lightwave interactions causing the SPR band to appear in the visible and infrared region. Metallic nanoparticles are produced by various methods,the more common ones being chemical and physical methods.Though these methods produce pure and well-definednanoparticles, the chemicals used in the synthesis process are toxic, energy consuming, expensive, and not suitable forbiological applications. Heteroployacids (HPAs), polysaccharides, tollens, irradiation, and biological methods have been introduced for the green synthesis of nanoparticles [5, 6]. Biological methods using microorganisms [7, 8], enzymes [9] and plant or plant extracts [10] also addressed the challenge of preparing nanoparticles. Cyanobacteria are considered as better biological template for nano-scale particle synthesis, due to high growth rate, high biomass productivity, and different biological activities by nature. So far very few scientists have tried cyanobacteria (intracellular) as well as cyanobacterial extracts (extracellular) for nanoparticle synthesis [11, 12] and used for the production of Ag, Au, Pd, Pt nanoparticles. [13] have used *Plectonemaboryanum* UTEX 485 for the synthesis of Pt,Pd and Ag nanoparticles, where as *Oscillatoriawillei*NTDM01 [14]*Valderianum*,*Gleocapsa* sp. *Phormidium*sp. *Lyngbya* sp. and *S.platensis* [12, 15] were also used successfully for the synthesis of AgNP.Through biological means, large quantities of nanoparticles at low cost have been successfully synthesized [16].Silver nanoparticles have received much attention due to theirphysical, chemical, and biological properties that attributedto the catalytic activity and bactericidal effects and foundapplications in bimolecular detection and diagnostics [17], antibacterials [18], therapeutics [19] catalysis [20], biosensors [21], and in plant growth metabolism [22, 23, 24]. Theyare used as antimicrobial agents in wound dressings [25], as topical creams to prevent wound infections [27, 27] and as anticancer agents [28].Nanoparticles show completely new or improved properties, based on specific characteristics, such as grain size, distribution, morphology, andthe material used for the green synthesis if compared with larger particles of the bulk material from which they were made of. With this in view, the present study aimed at greensynthesis of silver nanoparticles with the crude extracts of *Calothrix membranacea* KLR006 and examined its antibacterial properties against various pathogenic bacteria.

2. MATERIALS AND METHODS

2.1 Cyanobacterial strains collection and cultivation

Our previous study reported on the collection ofcyanobacterial samples from various freshwater ponds, lakes and agricultural fields atShrirangam, Manachanallur, Thiruverumbur, Mathur and Vayalur [29]. The axenic cyanobacterial samples were cultivated in conical flasks with BG 11 medium [30] and incubated at 28 ± 2ºC with illumination at 25-30 µmol photon $m^{-2}s^{-1}$using white continuous light. Cyanobacterial specimens were identified based on the morphological

descriptions[31, 32]. The tested strain *Calothrix membranacea*KLR006 was harvested by centrifugation with distilled water before lyophilization.

2.2 Crude extract preparation

Dried axenic form of *Calothrix membrancea* KLR006 was grained by morter and pestlefor powder preparation and then mixed with 100 mL double distilled water in Erlenmeyer flask. The mixture was centrifuged at 4000 rpm for 10 min at 4°C. Finally, the crude extract was collected by filtration with Whatman no.2 filter and stored at 4°C for further use.

2.3 Green synthesis of silver nanoparticles

Cyanobacterial crude extract was added to a solution of $AgNO_3$ (Himedia, India) to make up final concentration of 1mM and kept at 30°C in the the presence of ±2000 lux light. 1 mM $AgNO_3$ solution without cyanobacterial biomass extracts were also kept in parallel under identical conditions as control. Synthesis of nanoparticles from cyanobacterial extract was noticed by the change in solution color (pale yellow to blackish brown).

2.4 Isolation of nanoparticles

To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, the solution with nanoparticles were centrifuged at 9000 rpm for 10 min and redispersed in 10 mL sterile distilled water (five times). Thereafter, the purified suspension was dried at 30 ºC for further characterization.

2.5 UV – VIS spectra analysis

The synthesis of pure silver ions was recorded by measuring the UV-vis spectra of the solution at room temperature with UV–VIS spectroscopy in the range of 300–700 nm(Labtronics LT-spectrophotometer) operated at a resolution of 1 nm as a function of reaction time.

2.6 SEM and EDAX analysis

Small amount of synthesized AgNPs were sprayed on 12 mm diameter round glass cover slips to make thin film and kept at hot air oven for drying. This thin film was then used for the SEM and EDAX analysis (JEOL, JSM-5610, Japan).

2.7 X-ray diffraction (XRD) analysis

The X -ray diffraction (XRD) pattern was obtained with a PW 1800 Philips diffractometer using Cu-Ka radiation (k = 0.1541 nm), and the data were collected from 10° to 8° (2Ɵ) with a scan speed of 4 min^{-1}.

2.8 FT- IR analysis

The FT-IR (infra red) spectra of silver nanoparticles was recorded using Automated Tensor 27 FT-IR Spectrometer (Brucker Co., Germany) in the Range of 400-4000 $cm^{-1}$and identification of various functional groups were done by using the KBr pellet technique as described previously [33].

2.9 Antimicrobial activity

Antimicrobial sensitivity was carried out to detect whether the synthesized silver nanoparticles has

antagonistic characters against pathogenic bacterial strains. *In vitro* antibacterial activity was performed using agar well diffusion technique [34] where Mueller Hinton agar plates were inoculated first with the freshly prepared bacterial suspension separately using sterile cotton swabs; then the plates were perforated using sterile cork borer of 6 mm diameter into equidistant wells to be filled with 70 µL of the cyanobacterial AgNPs and with the negative control of $AgNO_3$solution. Plates were incubated at 37°C for 24 h. Theantibacterial activity was determined by measuring theinhibition zone around each well respectively and the average of repeated experimentswere obtained. Data were compared with positive and negative controls, standard antibiotic discs and with $AgNO_3$ solution.

3. RESULTS AND DISCUSSION

The tested cyanobacterial strain *Calothrix membranacea* KLR006 was isolated from Kollidam River at Srirangam sampling site [29]. Under microscope bluish green long and curved filaments with thin and hyaline sheath was seen. The trichomes, 3.9 - 6.6 µ broad and the heterocysts 3.9 o 5.2 µ broad (Fig. 1). In the present study, *Calothrix membranacea* extract added to $AgNo_3$ solution turned yellowish brown colour after 7 hours of incubation. The reduction of silver ions was quite rapid. The visual colour change in to yellowish brown confirmed the formation of AgNp (Fig. 2). UV–vis spectroscopy is an important technique to determine the optical property and stability of synthesized nanoparticles. Absorption peaks between 440 and 490 nm showed the presence of AgNp in the suspension. SEM microphotograph was obtained with VEGA 3 Tescan SEM to visualize shape and size of biosynthesized AgNp. Cyanobacteria and algae involve biosynthesis approach which takes longer time in nanoparticle synthesis. Biosynthesis of AgNP (100–200 nm, spherical) were synthesized within 72 h in *Oscillatoria willei* NTDM [35]. In *Plectonema boryanum* UTEX 485 200 nm octahedral/ spherical AgNP were synthesized in 28 days (36). Isolation of intracellular AgNP is multistep process. It involves efficient cell disruption (sonication/enzymic/physic chemical) and separation of nanoparticles from rest of the cellular components. But recently, few workers have started utilization of extracellular cell free approach [37, 38]. This is easier and much better in terms of time and cost. Rate of reaction of extracellular synthesis is also very fast in comparison of intracellular synthesis. In this process aqueous cell extract act as reducing agent in silver nitrate solution for AgNP synthesis. In a present study using *Microchaete sp.* NCCU-342 aqueous extract took minimum time 30 h (40 nm, spherical) for AgNP synthesis *Chlorococcum humicola* produced spherical nanoparticles of 100 nm in 24 h [37]. The difference in nanoparticle synthesis potential of the cyanobacteria may be due to quantitative and qualitative differences in proteinaceous substances in the cell extracts.

Figure: 1 Photomicrograph showing the morphological features of *Calothrix membranacea* KLR006.

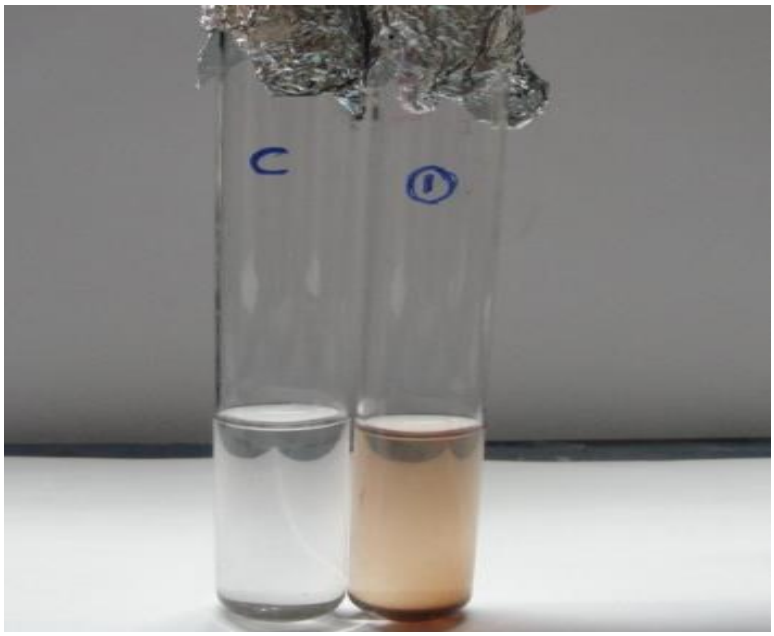

Figure: 2 Colour change from golden yellow to brownish-black observed when tested cyanobacterial strain extract was mixed with 1mM silver nitrate solution.

In terms of size, *Calothrix membranacea* KLR006 extracellular synthesized AgNP were 30–200 nm (Fig. 3) and polydispersity because of the biomolecules converting the surface of AgNps. In general smallest nanoparticles are considered better as they provide more surface area. Elemental composition analysis by energy dispersive X-ray (EDAX) analysis showed stronger signal from silver region (Fig. 4) and pure crystalline nature of the synthesized AgNps. The precipitation of silver nanoparticles was not observed for abiotic experiment that were run under similar condition and duration, suggesting that cyanobacterial extracts were required for silver precipitation presented for the reaction. FT-IR measurements indicate the biomolecules from *Calothrix membranacea* KLR006 were responsible for the silver ions reduction and stabilization of reduced silver ions

(Fig 5). The FT-IR spectrum of the green synthesized AgNPs from*Calothrix membranacea* KLR 006 showed strong absorption peaks at 3727.85, 3442, 2926.05, 1628.56, 1385.34 ,1275.44 and 671.41 cm-1 which represents the various functional group like OH stretching of alcohols or phenols, N-H group (amino acids), C-O of carboxylic anion, saturated C-O group and N-O stretching, respectively (Fig 5) X-ray diffraction analysis of synthesized AgNps are shown in (Fig 6) The diffracted intensities were recorded from 10 to Chloramphenicol and Methicillin, cefpodoxime and amoxicillin were used against gram positive and gram negative bacteria, respectively, as controls. Only the silver nitrate solution is added in the well for all the pathogenic microorganism as a negative control. There was minimum zone of inhibition shown for the tested organism. Whereas, the biosynthesized AgNPs added well showed a maximum inhibition zone against all of the pathogenic microbes tested except *P. aeruginosa.* Methicillin resistant *Staphylococcus aureus* showed (Table 1) resistant to the standard antibiotics chloramphenicol and methicillin whereas, 19 mm zone of inhibition was produced for the biosynthesized silver nanaoparticles indicating that biosynthesized AgNPs were potent in antibacterial agents even against drug resistance microbes. Similaraly *Klebsiella pneumoniae* showed resistant to standard antibiotics tested. A zone of inhibition of 24 mm was produced by biosynthesiszed silver nanoparticles indicating that *Klebsiella pneumoniae* is also susceptible. The effect of biosynthesized AgNPs on *P. aeruginosa* was intermediate only compared to the other pathogenic microbes tested.

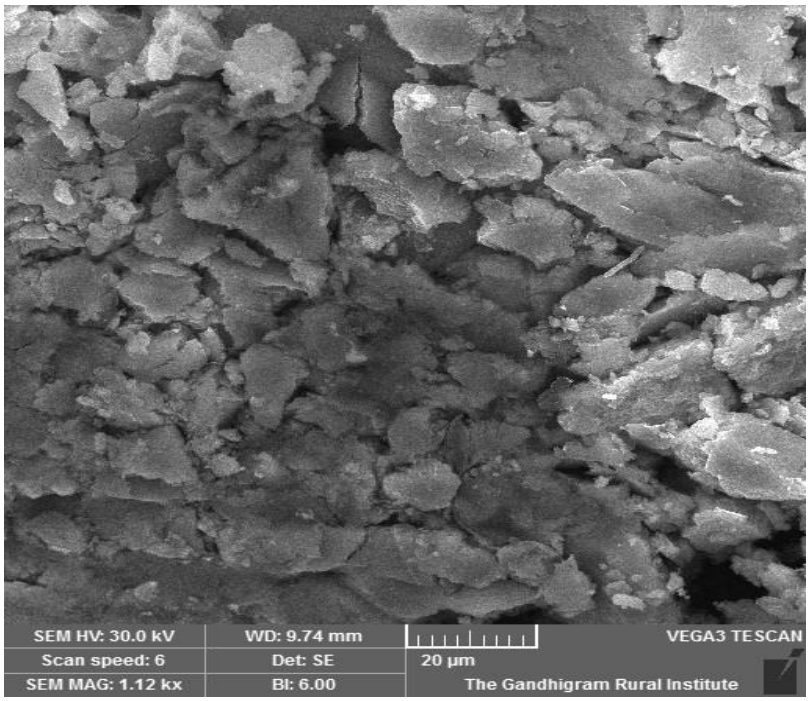

Figure: 3 SEM image showing biosynthesized AgNPs of polydispersity in shape and size.

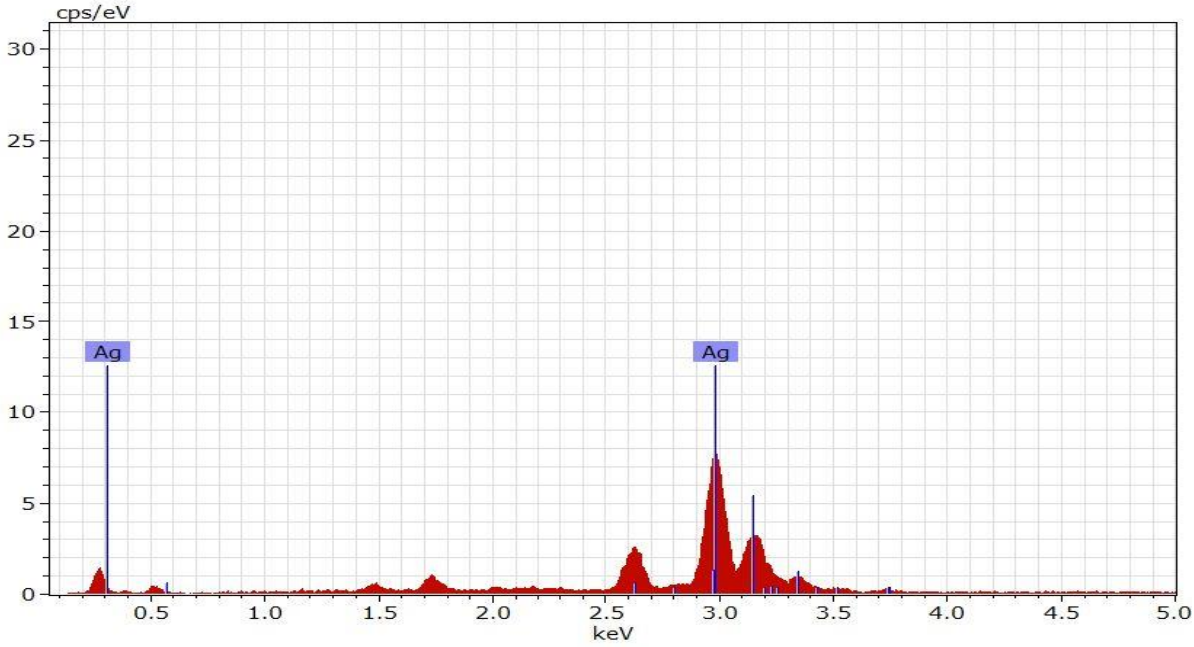

Figure: 4 EDAX spectrum of synthesized Silver nanoparticles

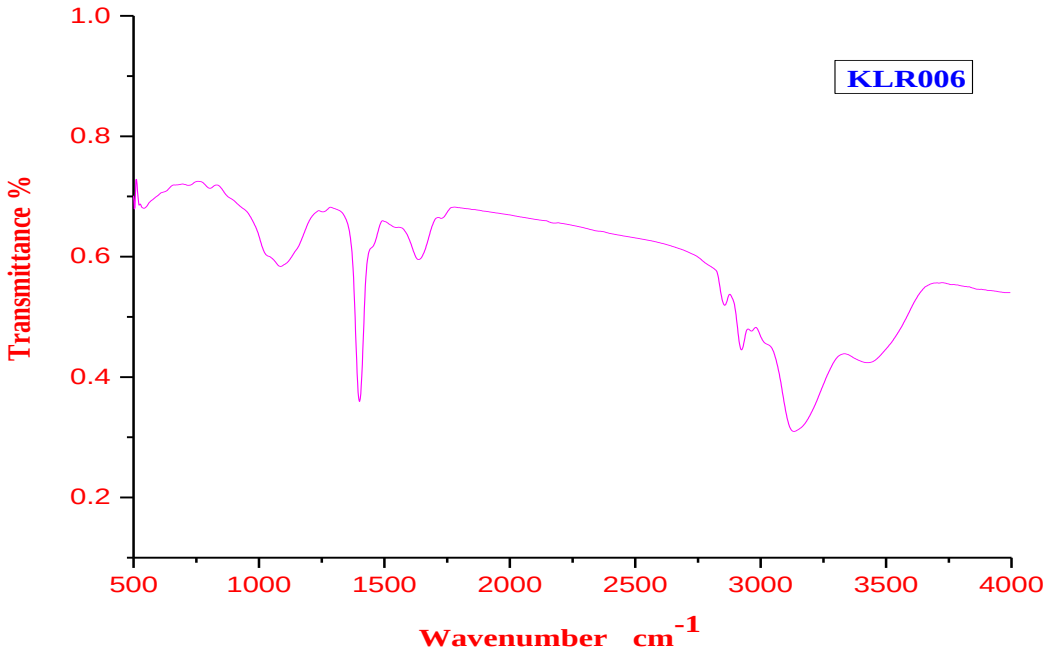

Figure: 5 FT-IR synthesized of silver nanoparticles

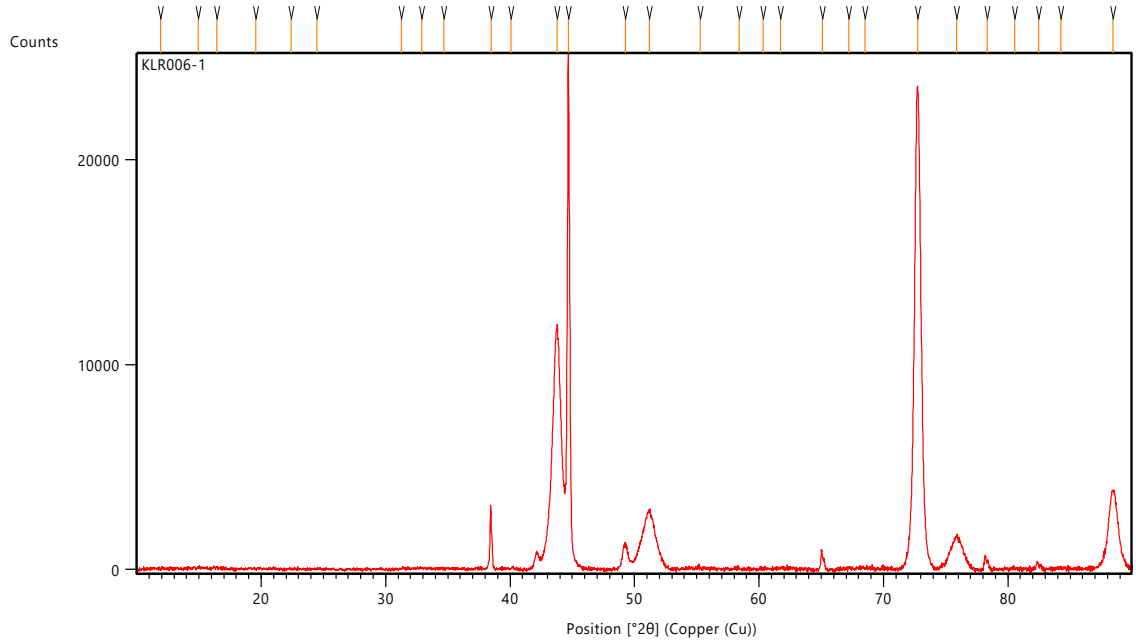

Figure: 6 XRD Analysis of *Calothrix membaranacea* KLR006 silver nanoparticles

Table: 1 Average inhibition zone of Ag particles synthesized measured in (mm)

Microorganisms	**$AgNo_3$ (Negative control)**	**Biosynthesized AgNPs**	**Standard Antibiotics (G + ve) bacteria**		**Standard Antibiotics (G-ve bacteria)**	
			Chloramphenicol	**Methicillin**	**Cefpodoxime**	**Amoxicillin**
S. aureus	6	28	23	18	-	-
Methicillin resistant S.aureus (MRSA)	2	19	11	16	-	-
V. chloerae	5	26	-	-	22	20
E. coli	4	28	-	-	24	18
K. pneumonia	2	24	-	-	15	12
P. aeruginosa	0	19	-	-	18	15

The enhanced antibacterial effects of biosynthesized AgNPs were due to the interference with the bacterial growth signalling pathway by modulating tyrosine phosphorylation of putative peptides substrate critical for cell viability and division [39, 40]. It was already reported that the silver nanoparticles have an antimicrobial activity towards *S. aureus*and *E. coli* [39]. The oligodynamic effect of silver has antibacterial activity against microorganisms. The changes in the local electronic

structure on the surface of the smaller sized particles lead to the enhancement of their chemical reactivity leading to bactericidal effect [41].

4. CONCLUSION

Several researches have already reported to the synthesis of silver nanoparticles using cyanobacteria. The extracellular synthesis of silver naoparticles using *Calothrixmembaranacea* KLR006 were obtained in the present study which showed potent antibacterial activity even towards drug resistant pathogenic microbes. It may be concluded that cell free biosynthesis of AgNP using cyanobacterial extracts is one of the better technique in terms of time and cost, which can be explored for further large scale AgNPs production in future.

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CONFLICT OF INTEREST

Authors declared there is no conflict of interest.

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