International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 23-06-2024, Accepted: 24-07-2024, Published: 09-08-2024
Volume 9, Issue 8, 2024, Page No. 24-30

Synthesis of clindamycin conjugated silver nanoparticles using *Plumbago Zeylanica* stem extract and their antibacterial effect against multidrug resistant pathogenic bacteria

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Abstract
The problem of multidrug resistance (MDR) against commonly used antibiotics is of growing concern globally. Novel and effective strategies are being employed to treat various disease causing MDR pathogens. In the present study, we have prepared silver nanoparticles (AgNPs) and antibiotic clindamycin conjugated silver nanoparticles (Cm-AgNPs) by green synthesis using aqueous *Plumbago zeylanica* leaf extract for their application to treat gram positive MDR pathogens. The synthesized NPs were characterized by UV-visible spectroscopy, Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FT-IR) and X-ray diffraction analysis (XRD). The UV-visible spectra of AgNPs and Cm-AgNPs show characteristic absorption peak at 440nm and 460nm respectively confirms the formation of NPs. The distinct SEM images of AgNPs and Cm-AgNPs show the formation of spherical particles without aggregation. The FT-IR spectrum show corresponding absorption bands of 3890cm^{-1} and 3000cm^{-1} confirms the AgNPs, and the presence of absorption band at 1100cm-1 confirms the conjugation of antibiotic. The XRD pattern obtained for the AgNPs and Cm-AgNPs show prominent Diffraction peaks with 2θ values confirms the crystalline nature of NPs. The antimicrobial activity of AgNPs and Cm-AgNPs were tested against three Gram positive MDR pathogens *Enterococcus faecalis*, *Enterococcus faecium* and *Staphylococcus aureus* by agar well plate method. Cm-AgNPs showed 7.14 %, 8 % and 8.33% more activity against *E. faecalis*, *E. faecium* and *S. aureus* respectively, compared to standard antibiotic Clindamycin. These results clearly demonstrate the utility and potency of Cm-AgNPs in combating MDR gram positive pathogens.

Keywords: Multidrug resistant. pathogenic bacteria. *plumbago zeylanica*. *enterococcus faecalis, enterococcus faecium* and *Staphylococcus aureus*

Introduction

The term “nano” is derived from a Greek word, nano means “dwarf”. In broad sense “Nanotechnology is the study of all the phenomena and process involved the synthesis, properties and application of nanostructures and nano materials” [1]. Nanoscale materials are defined as a set of substances where at least one dimension is less than approximately 100 nanometers or nanomaterials are one millionth of a millimeter approximately 100,000 times smaller than the diameter of a human hair. Nanoparticles have all three dimensions on the nano scale. Nanoparticles can also be embedded in a bulk solid to form a nanocomposite [2]. Although the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, therapy, and drug-delivery vehicles, bionanotechnology research is still in its infancy [3]. The nanoparticles are generally classified into three types, they are Organic nanoparticles, Inorganic nano particles and Carbon based nanoparticles. Some of the examples for organic nanoparticles were Dendrimers, Micelles, liposomes and ferretin [4]. Inorganic nanoparticles are particles that are not made up of carbon. Metal and metal oxide based nanoparticles are generally classified as inorganic nanoparticles. Particularly, silver nanoparticles (AgNPs) have received more attention due to their physical, chemical, and biological properties that are attributed to their catalytic activity and bactericidal effects. They are used as antimicrobial agents in wound dressing, as antiseptic lotion to prevent wound infections, and as anticancer agents. The antimicrobial potency of silver increases with increased surface area. The biological activity of AgNPs depends on number of factors such as their surface chemistry, size, size distribution, particle morphology, shape, particle composition, coating or capping, agglomeration, dissolution rate, particle reactivity in solution, efficiency of ion release, and cell type [5].

The White leadwort, *Plumbago zeylanica* is a medicinal plant commonly called “White leadwort” in English and “Chitrak” in Sanskrit, it belonging to the *Plumbaginaceae* family. It is a perennial herb which is found in West Bengal, Uttar Pradesh and Maharashtra and also to some parts of South India [6, 7]. It is having multi-purpose medicinal property and is most commonly used in the traditional medicinal system of India. The plants consist of various bioactive compounds like alkaloids, flavonoids, naphthoquinones, glycoside, saponins, steroids, triterpenoids, coumarins, phenolic compounds, tannins, carbohydrates, fixed oils, fats and proteins present in different parts of the plant which have been reported to show anti-bacterial, anti-plasmodial, anti-tumour, hepatoprotective, central nervous system stimulatory activity, anti-fungal, anti-inflammatory, anti-hyperglycemic, anti-cancer and anti-atherosclerotic activity [8]. The major chemical components of *Plumbago-zeylanica* consists of the Chitranone, Plumbagin, 3-Chloroplumbagin, droserone, Elliptinone, Zeylanone & Zeylinone, Maritone, Plumbagic acid, Dihydrosterone, B-sitosterol, etc. *Plumbago-zeylanica* is very popular throughout Africa and Asia as a remedy for skin diseases, infections and intestinal worms, especially leprosy, scabies, ringworm, dermatitis, acne, sores, ulcers of the leg, haemorrhoids and hookworm. It also helps in digestion [9].

Fig 1: *Plumbago zeylanica*, White leadwort plant

Clindamycin was first prepared in 1966 from lincomycin. It is on the World Health Organization's List of Essential Medicines. It is available as a generic medication. In 2019, it was the 119th most commonly prescribed medication in the United States, with more than 5 million prescriptions [10, 11].

Materials and methods

Sample collection and extract preparation

White leadwort stem was collected from the Kuvempu University garden, Shankaraghatta, Karnataka, India. 50 gm of White leadwort stem was powdered using pestle and mortar. The powder was boiled with 500 ml distilled water in 1000 ml beaker for 30 min. The extract was cooled and filtered twice through Whatman no.1 filter paper to get clear solution. The filtrate was stored in 500ml Erlenmeyer flask at 4°c for further experimental use [12].

Preparation of silver nanoparticles (AgNPs)

Silver nitrate solution (1mM) was prepared by adding 0.017gm in 100ml of distilled water. The *Plumbago Zeylanica* aqueous stem extract was mixed with silver nitrate solution in the ratio of 1:3. This mixture was mixed thoroughly and incubated for 24 hours in dark condition. After incubation, the formation of AgNPs was confirmed by measuring the UV-Visible absorption spectra from 300-600nm using UV-visible spectrophotometer (Eppendorf) [13, 15].

Preparation of Clindamycin conjugated silver nanoparticles (Cm-AgNPs)

The antibiotic Clindamycin (1%), silver nitrate solution and *Plumbago Zeylanica* stem extract were mixed in the ratio of 1:3:1. This mixture was incubated for 24 hours under dark condition. After incubation, the formation of Cm-AgNPs was confirmed by measuring the UV-visible absorption between 300-600nm using UV-visible spectrophotometer (Eppendorf) [16, 18].

Characterization of silver nanoparticles

UV-visible spectroscopy

The optical properties of synthesized silver nanoparticles were determined by using UV-visible spectrometry. The characteristic UV-visible absorption peak can be observed in the range 350 to 450nm of AgNPs and Cm-AgNPs [19, 20].

Scanning electron microscopy (SEM)

The SEM analysis is the best method for determining the surface topography and 3D view of the synthesized nanoparticles. The morphological characteristics of AgNPs and Cm-AgNPs were established by SEM. Thin films of the sample were prepared on a carbon coated copper grid by dropping a very small amount of the sample on the SEM grid and the film was allowed to dry by keeping it under a mercury lamp for 5 min and then subjected for SEM analysis [21, 22].

Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectra were used to identify the probable biomolecules present in the plant extract which are responsible for the reduction of metal ions. FT-IR measurements were carried out to identify the major functional groups in the *Plumbago Zeylanica* stem extract and their possible involvement in the synthesis and stabilization of silver nanoparticles [23, 24].

X-ray diffraction studies (XRD)

The size of particle and nature of silver nanoparticles were resolved by XRD. A thin film of the dried silver nanoparticles was coated on an XRD grid and XRD patterns were studied. The obtained data which is helpful for analysis having peak corresponding to different planes of crystal. The average size of crystalline silver nanoparticles was calculated from the width of the peaks [25, 27].

In vitro antimicrobial of AgNPs and Cm-AgNPs

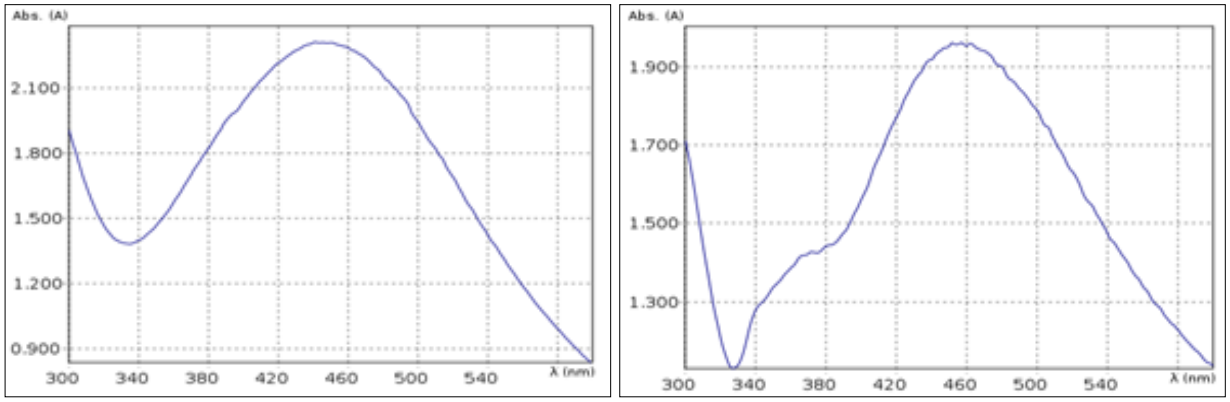
The antibacterial activity of AgNPs and Cm-AgNPs was investigated by agar well plate method, *in vitro*, along with antibiotic clindamycin as control. The cultures of *Enterococcus faecalis, Enterococcus faecium* and *staphylococcus aureus* were spread uniformly on solidified nutrient agar in different petriplates. The synthesized AgNPs, Cm-AgNPs, Clindamycin and *Plumbago Zeylanica* stem extracts were added to wells made in these solidified nutrient media and the plates were incubated at 30^0C for 16-24 h for the visualization of inhibition zones. The inhibition by the antibiotic clindamycin was considered as control. The tests were performed in triplicates and the data obtained was statistically analyzed by ANOVA [28, 30].

Results

Characterization of AgNPs and Cm – AgNPs

The addition of clindamycin during the synthesis of AgNPs results in the adsorption on to the surface of AgNPs. This can be attributed to weak electrostatic interaction between s-atom of clindamycin and Ag atom in the AgNPs [31].

UV-visible analysis

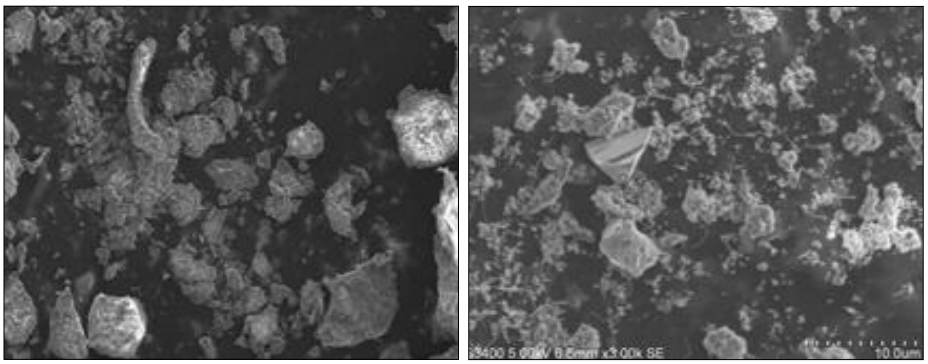


a) b)

Fig 2: UV-visible absorption spectra of a) AgNPs and b) Cm-AgNPs synthesized using *P. zeylanica* stem extract

The UV- visible absorption spectra was used for the structural characterization of synthesized AgNPs. The absorption band in the range 350 to 550 nm region is typical for the AgNPs. The UV-visible spectra of AgNPs and Cm-AgNPs show (Fig. 3) characteristic absorbance peak at 440 nm and 460 nm respectively.

Scanning electron microscopy (SEM) analysis

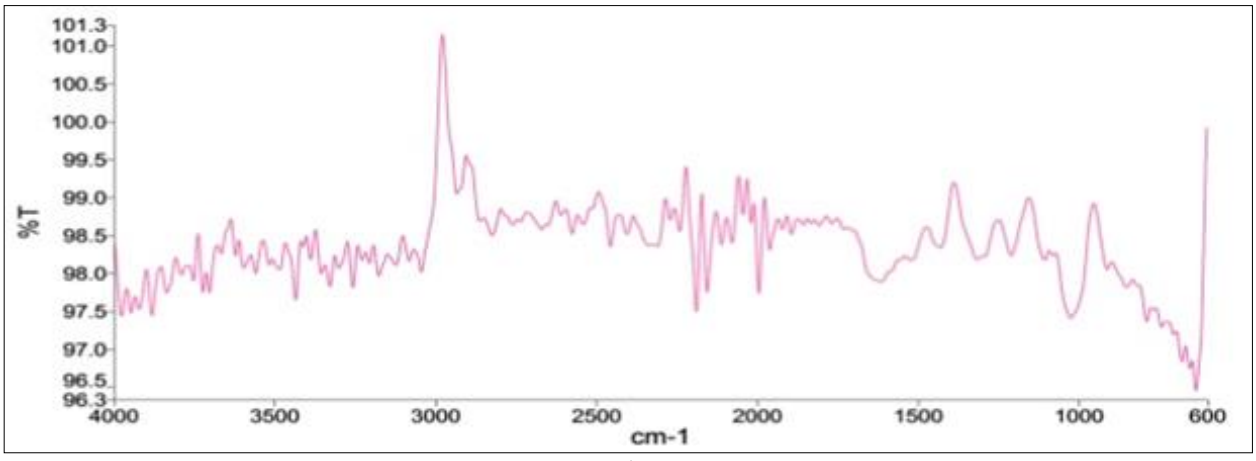


a) **b)**

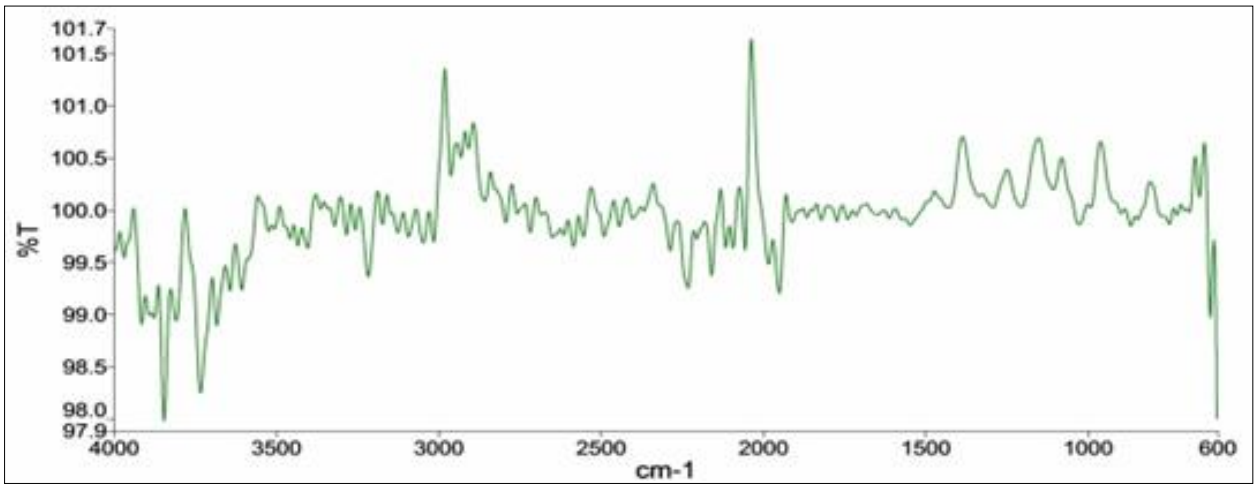
Fig 3: SEM images of a) AgNPs and b) Cm-AgNPs synthesized using *P. zeylanica* stem extract

The microscopic surface features including morphology and particle size of synthesized AgNPs and Cm-AgNPs were assessed by SEM images [32]. The nanoparticles were found to be irregular in shape with a diameter ranging from 24 to 26 nm and 28 to 32 nm, respectively (Fig 4). The SEM images also confirm that the synthesized nanoparticles were well separated with no aggregation.

Fourier transform-infrared spectroscopy analysis



a)

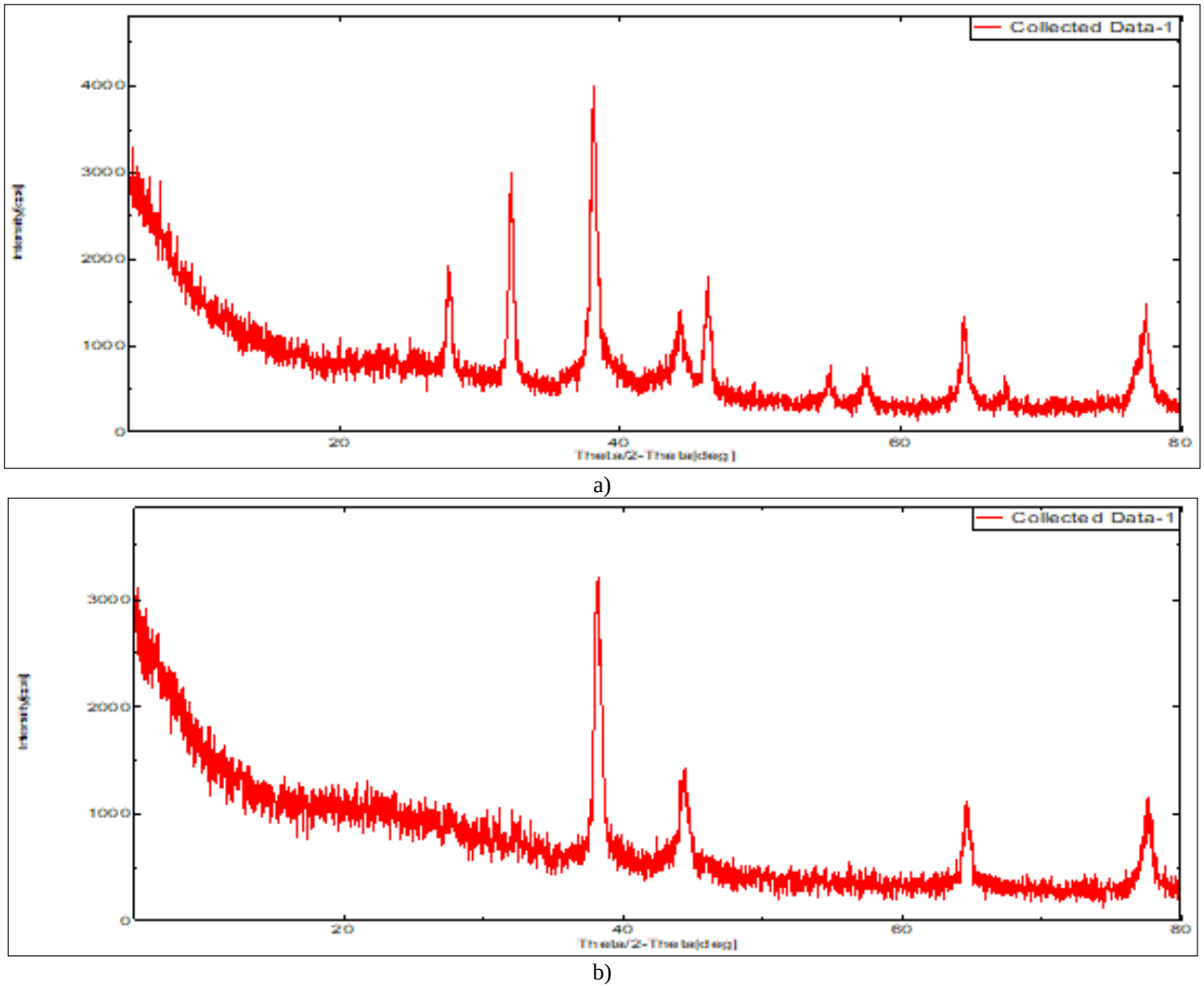


b)

Fig 4: FT-IR spectra of a) AgNPs and b) Cm-AgNPs synthesized using *P. zeylanica* stem extract

FT-IR measurements were carried out to identify the possible biomolecules responsible for the capping and efficient stabilization of silver nanoparticles synthesized by the stem extract shows several peaks indicating the complex nature of the biological sample [33, 34]. The absorbance bands of AgNPs synthesized from *P. zeylanica* stem extract and conjugated AgNPs with clindamycin were observed at 3890cm^{-1} assigned to O-H stretch, 3000cm^{-1} showed the stretching vibration of N-H and O-H groups and the absorption bands at 1064cm^{-1}, 1116cm^{-1} and 1002cm^{-1} correspondence to C=O, C=C, and C-O groups, respectively. In addition, the presence of absorption band at 1100cm^{-1} confirms the presence of C-OH stretch.

X-ray diffraction analysis



a)

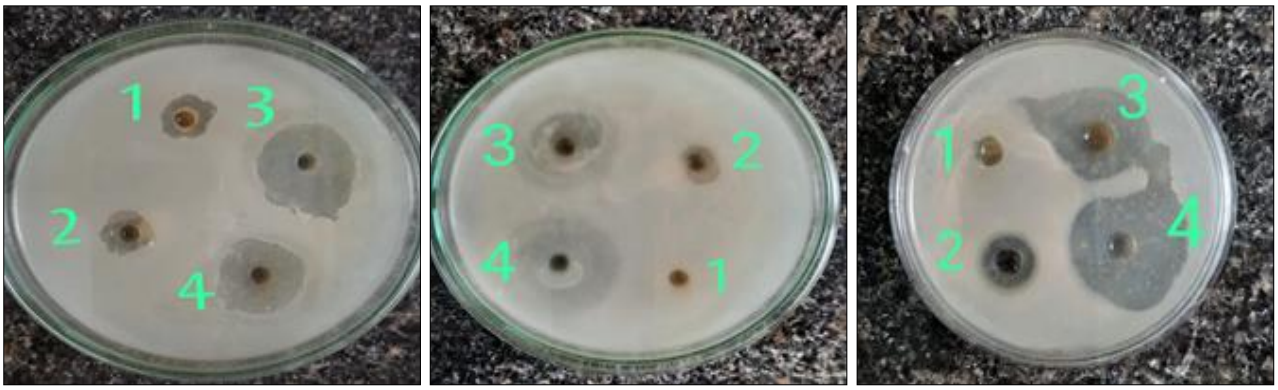
b)

Fig 5: The XRD pattern of a) AgNPs and b) Cm-AgNPs synthesized using *P. zeylanica* stem extract

The XRD pattern obtained for the AgNPs shows prominent diffraction peaks with 2θ values of 27.90º, 32.23º, 38.14º, 44º, 46.21º, 64.52º and 77.44º which are indexed as crystalline silver. The XRD pattern of Cm-AgNPs showed characteristic peaks of 2θ at 38.17º, 44.36º, 64.60º, 77.61º.

Antimicrobial activity of AgNPs and Cm-AgNPs

The antimicrobial potential of AgNPs and Cm-AgNPs was assessed against *E. faecium*, *E. faecalis* and *S. aureus.* The results showed that the inhibition of microbial growth was observed with both AgNPs as well as Cm-AgNPs. Inhibition of microbial growth by the white leadwort stem extract, silver nanoparticles, clindamycin conjugated silver nanoparticles and Clindamycin antibiotic was observed (Fig 7). Cm-AgNPs showed 7.14%, 8% and 8.33% more activity compare to standard Clindamycin against *E. faecalis*, *faecium*, and *S. aureus*, respectively [35. 37] (Table 1).



a. *E. faecium* **b.** *E. faecalis* **c.** *S. aureus*

Fig 6: Antimicrobial activity of (1) White leadwort extracts, (2) AgNPs, (3) Cm-AgNPs and (4) Clindamycin antibiotic for different microorganisms.

Table 1: The inhibitory effect of AgNPs and Cm-AgNPs on the growth of *E. faecalis*, *E .faecium* and *S. aureus*

Name of microorganisms	Zone of inhibition (cm)			
	White leadwort extract	AgNPs	Cm-AgNPs	Clindamycin
Escherichia faecalis	1.4±0.1	1.6±0.1	3.0±0.1	2.8±0.1
Escherichia faecium	1.2±0.1	1.4±0.1	2.7±0.1	2.5±0.1
Staphylococcus aureus	1.1±0.1	1.3±0.1	2.6±0.1	2.4±0.1

Discussion

Nanotechnology deals with the application of nanoparticles in biological, chemical, physical, environmental, agricultural, industrial or pharmaceutical science. Nanotechnology will have a deep impact on disease prevention diagnosis and treatments. Silver nanoparticles possess unique properties with a wide range of application such as antimicrobial, anticancer, catalyst and wound healing activities. The synthesized silver nanoparticles are subjected for characterization by UV-visible spectroscopy, Scanning electron microscopy, X-Ray diffraction, FT-IR spectroscopy. The UV-visible spectroscopy is a primary confirmation of the presence of silver nanoparticles. UV-visible spectra showed the characteristic absorption peak at 440nm which confirms the formation of AgNPs and Cm-AgNPs shows peak at 460nm. SEM revealed a uniform alignment of silver nanoparticles having size in the range of 24-26nm and 28-32nm respectively. The SEM images also confirm the synthesized nanoparticles and were well separated with no aggregation.

Antibacterial activity refers to the process of killing or inhibiting the disease causing microbes. Various antimicrobial agents are used for this purpose. Antimicrobial may be anti- bacterial, anti-fungal or antiviral. They all have different modes of action by which they act to suppress the infection. Antibiotics are used against bacteria, and antifungal are used against fungi. They can also be classified according to their function. Agents that kill microbes are microbicides, while those that merely inhibit their growth are called bacteriostatic agents. The use of antimicrobial medicine to treat infection is known as antimicrobial chemotherapy, while the use of antimicrobial medicines to prevent infection is known as antimicrobial prophylaxis. Antimicrobial activity can be determined by Agar disk diffusion method. Agar disk-diffusion testing developed in 1940, is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. In this well-known procedure, agar plates are incubated with a standardized inoculum of the test microorganism. Then, filter paper disc (about 6mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured. [39-42].

The results of antimicrobial activity studied by Agar well diffusion method showed a significant inhibition by Cm-AgNPs when compared with standard antibiotic (Clindamycin). The data clearly indicates that the AgNPs conjugated with Clindamycin greatly enhance the antimicrobial activity of the silver nanoparticles. The Cm-AgNPs shows 7 % more activity when compare to Clindamycin. These conjugated AgNPs can potentially be used as economical and eco friendly. The antibacterial efficacy of the synthesized Silver nanoparticles was assed against *E. faecium*, *E. faecalis* and *S. aureus.* The result showed that the inhibition of microbial growth was observed with both AgNPs as well Cm-AgNPs. The results

clearly demonstrate the utility of Cm-AgNPs in enhancing the antibacterial potency of both Clindamycin as well as AgNPs. These results clearly demonstrate the utility of Cm-AgNPs in combating the effect of MDR gram positive pathogens.

Acknowledgements
The authors would like to thank the Department of Biochemistry, Kuvempu University, Shankaraghatta, karnataka, India for providing the laboratory facility.

Conflicts of interest/financial disclosures
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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