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Phytomediated synthesis and characterization of silver nanoparticles from the leaf extracts of *Clausena anisata* (Willd.) Hook. F. Ex Benth. and its antimicrobial activity

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Abstract
Development of biologically inspired phytomediated synthesis of silver nanoparticles is evolving into an important branch of nanobiotechnology. In the present investigation, we report the phytomediated synthesis of silver nanoparticles (AgNPs) employing the leaf extract of *Clausena anisata* (Willd.) Hook.f. ex Benth (Rutaceae). The synthesized Ag-NPs were characterized by UV-visible, X-ray diffraction (XRD), Fourier-transform infrared (FT-IR), Scanning electron microscopy (SEM), and Energy dispersive X-ray (EDX). Formation of silver nanoparticles was confirmed by the change of colour from pale yellow to dark brown in colour. These results authenticated that the appearance of AgNPs was analyzed by UV- visible spectrum around the peak 420 nm. XRD (X-ray diffractometer) demonstrated the formation of crystalline AgNPs with FCC structure having an average crystalline size of 20.42 nm from XRD profile. FT-IR analysis revealed the presence of different functional groups in the synthesized AgNPs. Antimicrobial activity of the synthesized silver nanoparticles was evaluated against Gram positive and Gram negative bacteria such as *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and fungus *Candida albicans*. Both the leaf extract and synthesized silver nanoparticles from the leaves of *Clausena anisata* showed moderate antimicrobial activity.

Keywords: phytomediated synthesis, characterization, *clausena anisata*, silver nanoparticles, antimicrobial activity

Introduction

Nanotechnology deals with the production and stabilization of various types of nanoparticles (Feymen, 1991) [6]. In order to obtain nanoparticles in large quantities within a short period, physical and chemical procedures are used (Bigall and Eychmuller, 2010) [5]. Biologically synthesized silver nanoparticles (Ag-NPs) have wide range of applications because of their remarkable physical and chemical properties (Balantrapu and Goia, 2009) [2]. Nowadays research mainly based on advanced nanomaterials of noble metals like silver has attracted a lot of interest among scientists during the past decades for its physiochemical properties such as size, distribution and morphology, they have been studied for catalytic activity, optical properties, electronic properties, antibacterial properties and magnetic properties (Song and Kim, 2009; Santos *et al* 2012) [15, 14] and its application in various field such as biomaterial production, biochemistry, medical and pharmaceutical products, toothpastes, optical receptors, biosensing, etc. (Banerjee *et al* 2014; Navaladian *et al.* 2007; Rajasekharreddy *et al.* 2010) [3, 12, 13].

Silver nanoparticles of range between 1 nm and 100 nm in size and have attracted intensive research interest. It is generally recognized that silver nanoparticles may attach to the cell wall, thus rupturing cell-wall permeability and cellular respiration. The nanoparticles may also penetrate inside the cell causing damage by interacting with phosphorus and sulfur containing compounds such as DNA and protein. Generally, silver does not adversely affect viable cells and does not easily provoke microbial resistance (Srividhya *et al.* 2018) [16]. Very recently plant extract of *Neolitsea sericea* (Srividhya Pattabiraman *et al.* 2018) [16], *Hugonia mystax* L (Tamilsevan *et al*. 2016) [17], *Corchorus tridens* (Karuppasamy *et al.* 2019) [10], *Abutilon indicum* (Ashokkumar *et al*. 2013) [1] reported in literature with nanoparticle size ranging from 5 to 20 nm are brimming in literature as a source for the synthesis of silver nano silverparticles as an alternative to the conventional methods. It is evident from the previous reports that no work has been carried for the synthesis, characterization and antimicrobial assay of synthesized silver nanoparticles from the aqueous leaf extracts of *Clausena anisata* (Willd.) Hook.f. ex Benth. By considering the vast potentiality of plants as sources, the present study was envisaged to apply a biological green technique for the synthesis of silver nanoparticles as an alternative to conventional methods.

Materials and Methods

Preparation of *Clausena anisata* leaves extract

The AR grade of silver nitrate was purchased from Sigma-Aldrich chemicals in India. Microbial strains were procured from Department of Biology, Gandhigram Rural Institute - Deemed University, Gandhigram. Mueller–Hinton broth and agar were purchased from Hi-Media, Mumbai, India and fresh leaves of *Clausena anisata* were collected from Pothigai hills, Tenkasi District, Tamil Nadu, India. 25g of the fresh leaf powder was mixed with 100 ml of double distilled water and transferred into the 500 ml beaker and boiled at 100°C for 40 minutes and then brought down to room temperature. Further, the extract was filtered with Whatman No.1 filter paper and stored at 4°C.

Microorganisms

The microbial strains such as bacteria *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Streptococcus faecalis* (MTCC 5383), *Klebsiella pneumoniae* (MTCC 4030), *Pseudomonas aeruginosa (*MTCC 741*),E.coli* (MTCC 443) and fungal *Candida albicans* (GRIBI 03) were collected from Department of Biology, Gandhigram Rural Institute - Deemed University, Gandhigram. Microbial strains were incubated at 37^0C in Mueller- Hinton Broth.

Synthesis of Silver nanoparticles using *Clausena anisata* leaves extract

10 ml of leaf extract of *Clausena anisata* was added to 1mM $AgNO_3$ (90 ml) aqueous solution. The reduction process of Ag^+ to Ag^0 was followed by the change of colour pale yellow to dark brown in colour which indicates the formation of AgNPs.

Characterization of AgNPs

The absorption spectrum of the synthesized silver nanoparticles from the leaves of *Clausena anisata* was analysed by UV Visible spectroscopy-1800v (Shimadzu, Japan) at the wavelength ranging from 200-700 nm. The external morphology of the synthesized silver nanoparticles was observed on scanning electron microscope (SEM) at GRI and elemental composition of synthesized AgNPs was confirmed by EDAX analysis. FT-IR spectra revealed that perkin Elmer spectrum 400 FT-IR transmission mode 4000-400 cm^{-1}. DLS and Zeta potential measurements were carried out on same instrument HORIBA SZ-100 which was used to evaluate particle size and surface charge of the synthesized AgNPs. The average grain size was calculated by image Joint Committee on Powder Diffraction Standards (JCPDS) software. The crystalline structure of the synthesized AgNPs was illustrated that XPERT-PRO through powder X-ray diffraction using Cu Kα radiation with and theta angles at 10º C to 80º C.

Antimicrobial activity

Antimicrobial activity of the synthesized silver nanoparticles was carried out by using *Bacillus subtilis* (GRIBI 01), *Staphylococcus aureus* (GRIBI 05), *Streptococcus faecalis* (GRIBI 08), *Klebsiella pneumoniae* (GRIBI 02), *Pseudomonas aeruginosa (GRIBI04), E.coli* (GRIBI 06) and *Candida albicans* (GRIBI 03) agar well diffusion method. Mueller hinton agar medium 30.4g MH medium was mixed with 800 ml of distilled water and sterilized in autoclave at 20 minutes. The sterilized medium was allowed to pour into the Petri dishes. The solidified plates were poured to Cork borer with the help of 6 mm diameter. The plates were plant extract, silver nitrate and different concentrations of synthesized AgNPs 100µg/ml, 150µg/ml, and 200µg/ml for antimicrobial studies.

Phytochemical analysis

The collected plant leaves extracts were subjected to synthesis of silver nanoparticles and synthesized particles were used in phytochemical analysis. (Table 1)

Test for Alkaloids (Mayer's Test)

One ml of synthesized AgNPs solution was mixed with 6 drops of mayers reagent. Yellowish creamish precipitate was formed and thus indicated the presence of alkaloids (Yadav *et al*. 2011; Kokate, 2000) [18, 11]

Test for Tannins (Braymer's Test)

One ml of synthesized AgNPs solution was mixed thoroughly with 2ml of water. To this mixture, two drops of 5% ferric chloride solution was added. Appearance of dirty green precipitate denoted the presence of tannins (Yadav *et al*. 2011; Kokate, 2000) [18, 11].

Test for Steroids (Salkowski Test)

Equal volume (2 ml) of the synthesised silver nanoparticles solution and chloroform was taken and added concentrated sulphuric acid along the side of the test tube. Formation of reddish brown ring at the junction gave positive result for steroids (Harbone, 1999) [8].

Test for Terpenoids

About 2ml of the solution was mixed with 2ml acetic acid. Then, few drops of concentrated sulphuric acid was added. The appearance of deep red color showed the presence of terpenoids ((Harbone, 1999) [8].

Test for Coumarins

About 2ml of the test solution was taken and mixed with 3ml of 10% sodium hydroxide. The formation of yellow colour denoted the existence of coumarins in the sample (Harbone, 1999) [8].

Test for Catechins

About 2ml of alcoholic test solution was treated with few drops of Echrilich reagent and few drops of concentrated Hydrochloric acid. The formation of pink colour denoted the presence of catcehin (Harbone, 1999) [8].

Test for Phenols

About 1ml of the test solution was treated with 3 ml of 3% aqueous ferric chloride solution. The appearance of deep blue colour gave positive result for phenol (Benzie and Strain, 1996; Hodges et al., 1999) [4, 8].

Test for Flavonoids

About 1ml of the test solution was treated with 1ml of sulphuric acid. The appearance of orange colour showed the presence of flavonoids (Benzie and Strain, 1996; Hodges et al., 1999) [4, 9].

Test for Quinones

About 1ml of the test solution was treated with 5ml of HCL. The formation of yellow colour precipitate showed the presence of quinine (Benzie and Strain, 1996; Hodges et al., 1999) [4, 9].

Test for Saponins (Foam Test)

About 1ml of the test solution was mixed with 5ml of distilled water. The contents were mixed well and heated in a boiling water bath for 10 minutes. Frothing indicated the presence of saponins (Harborne, 1973) [7]

Results and Discussion

Visual examination and UV-vis spectroscopy

Synthesis of silver nanoparticles was prepared by mixing of 20ml of aqueous leaf extract of *Clausena anisata* with 80ml of 1mM silver nitrate solution. Since the silver nanoparticles were formed from the mixture at room temperature, the colour of the mixture changed from brown into black in colour (Fig.1). The appearance of black colour indicated the bioreduction of silver ions into silver nanoparticles. The synthesis of silver nanoparticles from the aqueous leaf extract of *Clausena anisata* was monitored by UV-vis spectrophotometer in a range of wavelength from 400 to 600nm. The UV-vis spectroscopic studies revealed that the surface Plasmon resonance band was noted at 445nm. So, the silver nanoparticles were polydispersed. It was indicated that the absorption band at 445nm was closely attributed to the absorption band by the colloidal silver nanoparticles due to surface Plasmon resonance (Fig.2).

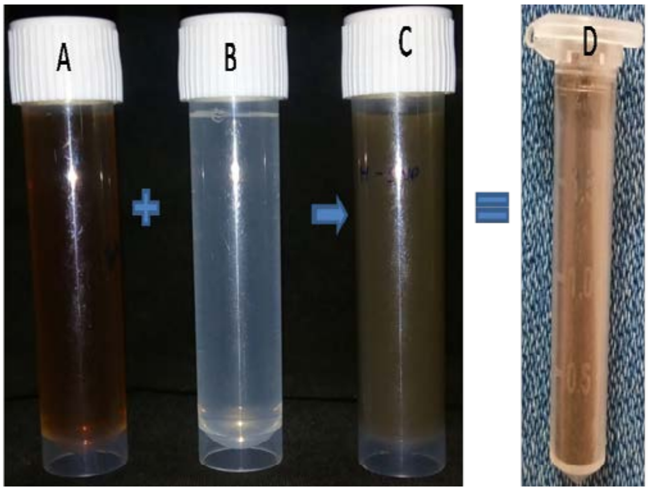

Fig 1: Change of color after the addition of silver nitrate solution in the Phytomediated synthesis of silver nanoparticles.

a. Plant extract
b. Silver nitrate
c. Plant extract with silver nitrate
d. silver nanoparticles

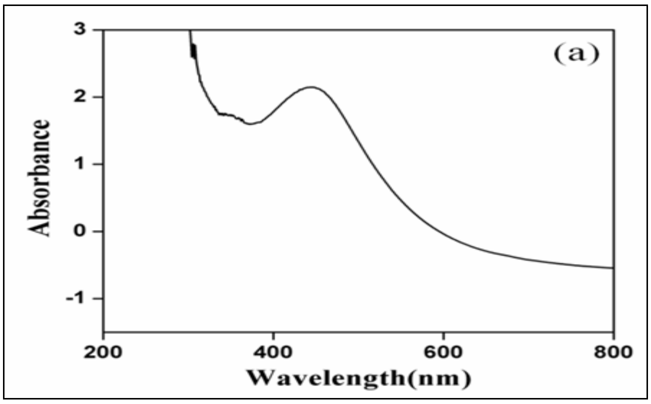

Fig 2: UV-visible spectrum of AgNPs synthesized using aqueous leaf extracts of *Clausena anisata*

FTIR analysis of synthesized AgNPs

FTIR has been used to detect the biomolecules which were involved in the green synthesis of silver nanoparticles of aqueous leaf extract of *Clausena anisata*. The FTIR spectra depicted the presence of certain functional groups which were visible at the wavelength ranging from 500 to 4000cm^{-1}. The spectrum imparted the broad peak in higher region at 3427.26cm^{-1}. The absorbance bands such as 1170.51 cm^{-1}, 1510.75cm^{-1}, 1800.54cm^{-1} and 3427.26cm^{-1} were assigned to C – O stretching, N – O stretching, C = O stretching and O – H stretching respectively. It is concluded that the groups such as O-H, C-H, N-H and alcohol were served as stabilizing agents thereby involving the reduction of silver ions to silver nanoparticles (Fig.3).

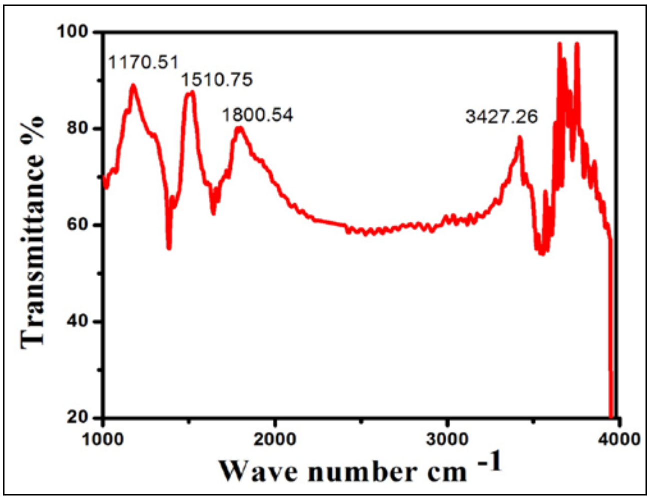

Fig 3: FTIR spectra of phytomediated synthesis of silver nanoparticles from the leaf extract of *Clausena anisata.*

X-ray diffraction study

The crystalline nature and quality of the silver nanoparticles were authenticated by XRD analysis. The XRD pattern of the phytomediated synthesized silver nanoparticles from the aqueous leaf extract of *Clausena anisata* was depicted in Fig. 4. The present study confirmed that the synthesized silver nanoparticles were crystalline in nature. In this case, the peaks were observed at 2θ of 23.50°, 33.81°, 38.08°, 64.38° and 77.35° which were attributing to crystallographic planes of Face Centred Cubic phase of Bragg's reflection such as (111), (200), (220) and (311). The data was perfectly correlated the standard diffraction data by Joint Committee on Powder Diffraction standards (JCPDS) File number 65-6811. The average particle size of the silver nanoparticles synthesized from the aqueous leaf extract of *Clausena anisata* was approximately 20.42nm.

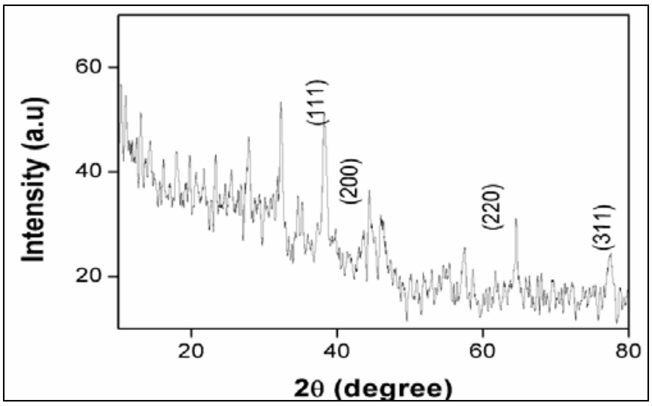

Fig 4: X-ray Diffraction profile of phytomediated synthesized silver nanoparticles by using leaf extracts of *Clausena anisata*

Scanning Electron Microscopy (SEM)

It is evident from the results that the size and morphology of the synthesized AgNPs was noted by SEM. Figure 5 shows that the synthesized nanoparticles consist of large number of spherical like structures with the length of micrometer (29µm).

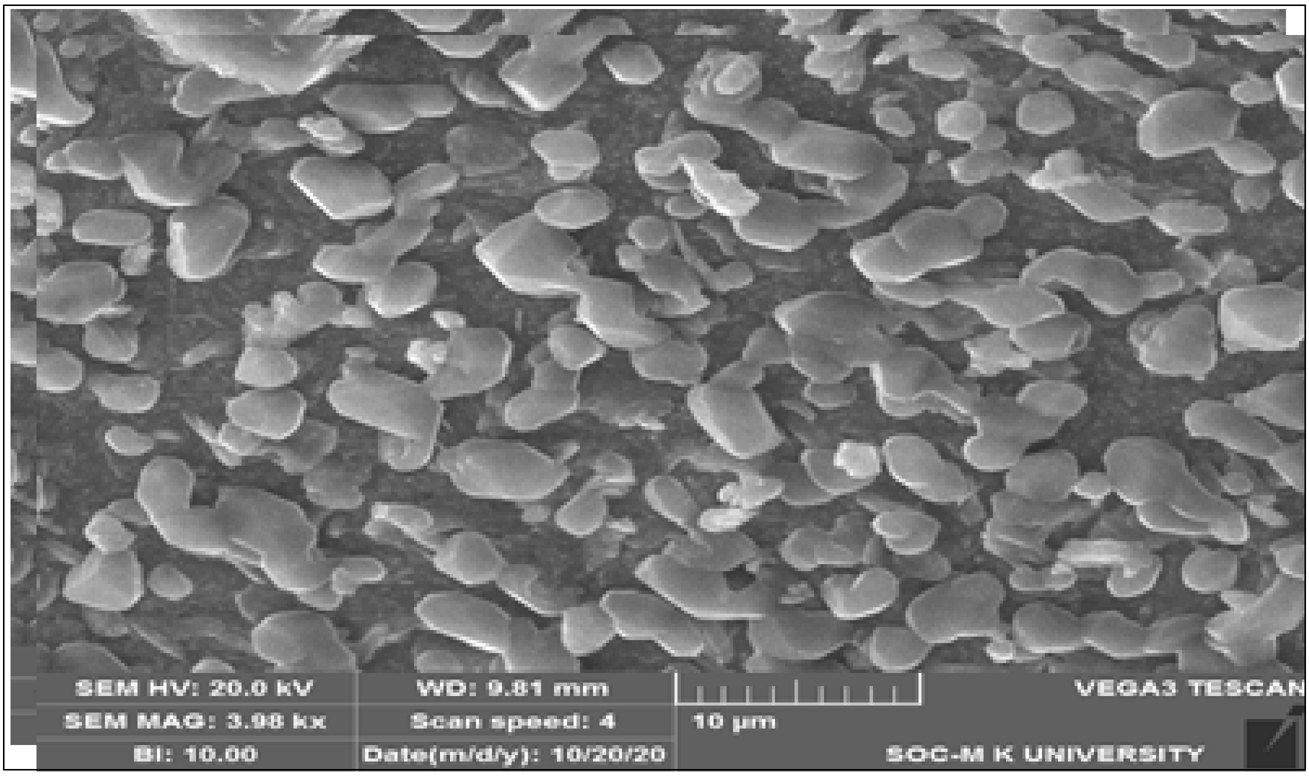

Fig 5: SEM image of phytomediated synthesis of Silver nanoparticles from the aqueous leaves extract of *Clausena anisata*

Energy Diffraction X-ray (EDX)

Energy Dispersive analysis by X-ray spectrum of synthesized silver nanoparticles by using aqueous leaf extract of *Clausena anisata* was presented in (Fig. 6). It is evident from the data that the X-ray spectrum exhibited the presence of elemental silver carbon and oxygen.

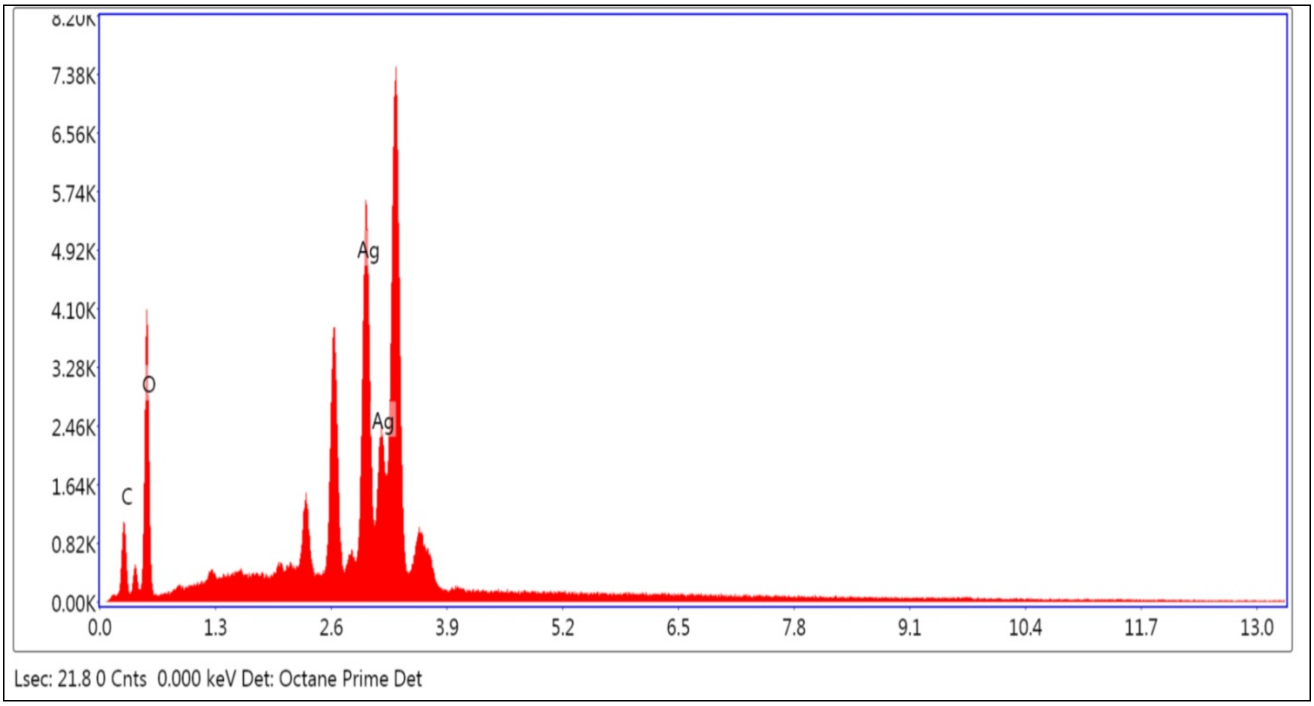

Fig 6: EDX phytomediated synthesis of silver nanoparticles from the leaf extract of *Clausena anisata*.

Qualitative Phytochemical Screening and Antimicrobial activity

The preliminary phytochemical screening of green synthesized silver nanoparticles when compared to the aqueous leaves extract of *Clausena anisata* revealed the presence of various phytoconstituents. The synthesized silver nanoparticles and aqueous leaf extract of *Clausena anisata* indicated the presence of the various phytochemicals such as alkaloids, coumarins, phenols, saponins and tannins. It is remarkable that the phytochemicals such as flavonoids, catechins, quinones, steroids, and terpenoids were not reported from the aqueous leaves extract of *Clausena anisata* and synthesized AgNPs. (Table 1).

Table 1: Preliminary phytochemical screening of aqueous leaves extract and synthesized AgNPs of *Clausena anisata*

Phytochemical Test	Aqueous leaves extract	Synthesized AgNPs
Alkaloids	+	+
Catechins	-	-
Coumarins	+	+
Flavonoids	-	-
Phenols	+	+
Quinones	-	-
Saponins	+	+
Steroids	-	-
Tannins	+	+
Terpenoids	-	-

+ indicates presence of phytochemicals; - indicates absence of phytochemicals

Antimicrobial activity

The antimicrobial activity of the green synthesized silver nanoparticles of *Clausena anisata* leaves had powerful antimicrobial activity against gram-positive, gram-negative and one fungal pathogens. The silver nanoparticles of three different concentrations (100µg/ml, 150µg/ml, and 200µg/ml) treated have antimicrobial activity against gram positive and gram negative bacteria, with varying various size of inhibitory zone. Moreover, the leaves extract of *Clausena anisata* showed lesser antimicrobial activity when compared to $AgNO_3$ and synthesized AgNPs. The results showed that AgNPs are effective antibacterial activity against *Bacillus subtilis* (20.01mm), *Klebsiella pneumoniae* (18.04 mm), *Candida albicans* (17.05 mm) and *E. coli* (18.04 mm) respectively. The remaining bacterial strains *Staphylococcus aureus* (14.08mm), *Streptococcus faecalis* (15.03mm) and *Pseudomonas aeruginosa* (14.07mm) are fairly susceptible against various concentration of silver nanoparticles. The AgNPs synthesized from the leaves of *Clausena anisata* exhibited strong antimicrobial activity against human pathogenic bacteria. (Plate 1).

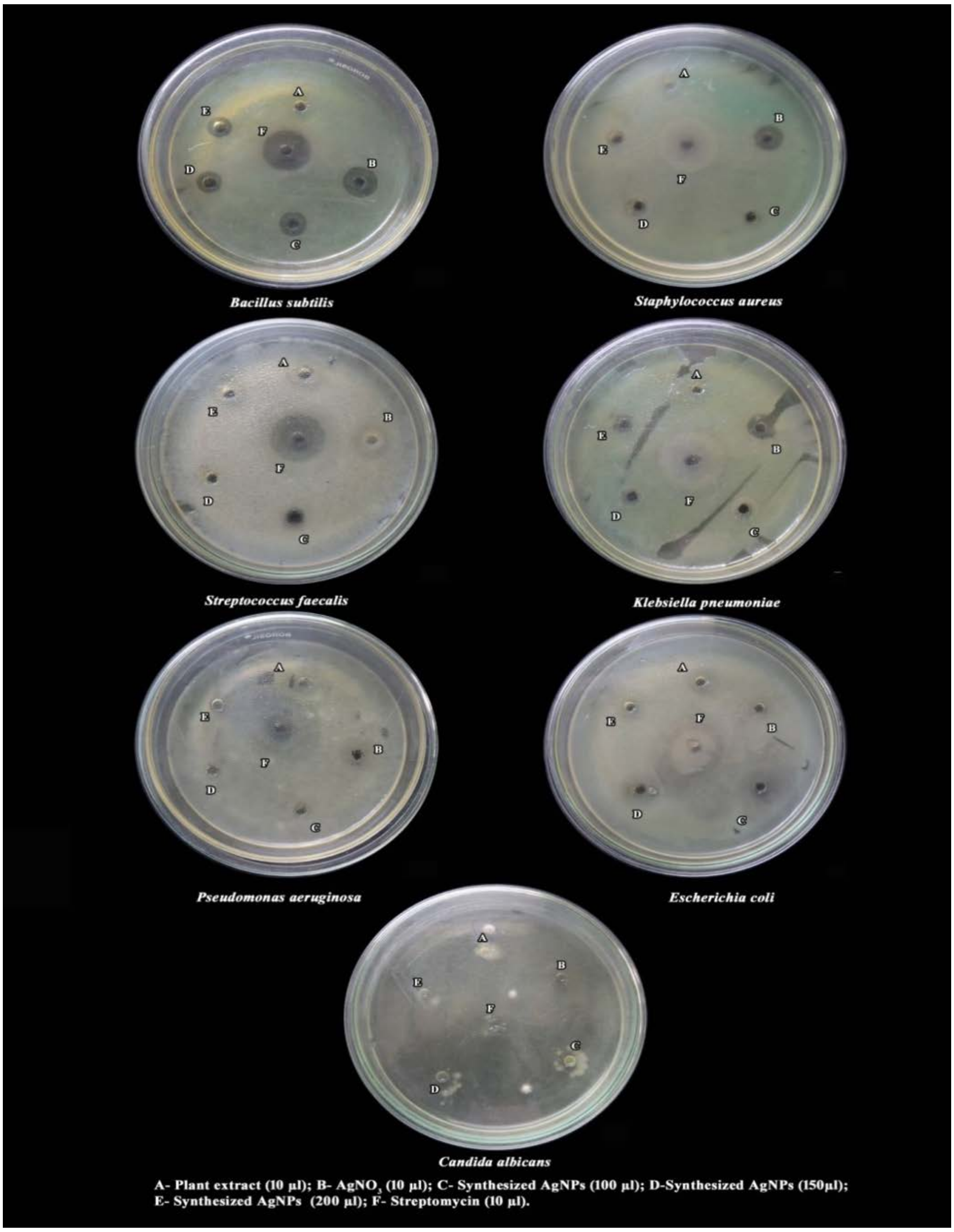

Plate 1: Antimicrobial activity of various concentration of synthesized Silver nanoparticles from the leaves of *Clausena anisata* L. against selective pathogens

Conclusions

The phytomediated synthesis of AgNPs using leaf extracts of *Clausena anisata* was shown to be rapid, eco-friendly and produced nanoparticles are fairly uniform in size and shape. The formation of silver nanoparticles started to form within 15minutes and higher formation yield at 45min after addition of leaf extract to silver nitrate as shown by the UV-vis spectrum at 445 nm. It was noticed that the formation of AgNPs depends upon the duration of incubation time. The XRD peaks ascribed with FCC structure of silver. The synthesized AgNPs were spherical in shape and particle size found about 20.42 nm from XRD results in addition justified further by the SEM analysis. The antimicrobial activity of the synthesized AgNPs was evidenced by different microbial strains such as *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E.coli* and fungal *Candida albicans*. Antimicrobial studies revealed that small size, spherical shaped nano particles have potential activity against different microbial strains and serves as eco-friendly antimicrobial agents. Therefore, this phytomediated synthesis of silver nanoparticles is more economic and beneficial to produce promising and compatible metal nanoparticles using biological method at commercial level and to explore their potentials in drug delivery system using surface capping activities of medicinally useful plant metabolites. Hence, it can be summarized that, Phytomediated synthesis of silver nanoparticles is an effective and eco-friendly method of producing metal nanoparticles.

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