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Green synthesis of silver nanoparticles using leaf extract of *Aegle marmelos* L. and its antimicrobial effect on human pathogens

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Abstract
In this present study *Aegle marmelos L.* was taken to investigate its potential for synthesizing silver nanoparticles. The silver nanoparticles synthesized from the ethanolic extract of leaves were confirmed by their change of colour to dark brown due to the phenomenon of surface plasmon resonance. The characterization of nanoparticle synthesized was done by UV-vis spectroscopy, Fourier Transmission infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), Dynamic light scattering (DLS) and X-Ray diffraction (XRD) studies. The medicinal plant mediated silver nanoparticles show good antimicrobial activity against clinically important pathogens *Staphylococcus aureus, Klebsiella* pneumoniae, *Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus pyogenes.*

Keywords: green synthesis, silver nanoparticles, SEM, FTIR, DLS, XRD

Introduction

Nanotechnology is a dynamically emerging field of modern research with possible effects in medicine (Chen, 2008, Boisselier and Astruc 2009) [4, 2]. Nanotechnology can be defined as a research for the synthesis, manipulation and design, the structure of particles with element size about 1-100nm. Metal mediated nanoparticles have a high precise surface area and a high fraction of surface atoms. Because of the distinctive physicochemical features of nanoparticles includes catalytic activity, optical and electronic properties, antibacterial potential and magnetic properties (Catauro *et al.,* 2005; Crabtree *et al.,* 2003) [3, 5] they are attained the interest of scientist for their innovative methods of synthesis. Over the past few years, the synthesis of metal mediated nanoparticles is an significant focus of research in modern material science. Different types of nanomaterials like copper, zinc, titanium (Retchkiman-Schabes *et al.* 2006) [17], magnesium, gold (Gu *et al*., 2003) [7] and alginate (Ahmad *et al.*, 2005) [1] exists but silver based nanoparticles evidenced to be most active since it has good antimicrobial effectiveness against bacteria, viruses and other eukaryotic micro-organisms. Silver nanoparticles are being used as a drug delivery system and an effective drug for cancer (Gong *et al.,* 2007) [6]. Silver nanoparticles shows higher antimicrobial activity that heals the wounds and also act against infectious disease (Ravishankar and Jamuna, 2012) [16]. Synthesis of nanoparticle becomes novel concern in nanotechnology due to its variability in size, shapes, chemical composition and controlled dispersity and their potential practise in the medical science for the enhanced treatment for human benefits.

Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al.* 2008) [13]. Plants were played a substantial role in sustaining human health and refining the quality of human life for thousands of years and have served humans as well as valuable source of new natural products. Despite the accessibility of diverse methods for the discovery of therapeutics, plant products still remain as one of the best reservoirs of new structural types. The secondary metabolites present in plant extracts such as flavonoids, alkaloids, phenolic compounds, terpenoids, etc., will reduce the metallic compounds into its nano compounds. For stabilization the active compounds will act as capping agents and enhance the activity of both nanoparticle and the therapeutic compound.

Due to the non-toxic, safe inorganic antibacterial agent of silver nanoparticles are being used for centuries and are capable of killing microorganisms which are cause for diseases (Jeong *et al.,* 2005) [9]. Silver has been designated as being "oligodynamic", which means its ions are accomplished of causing a bacteriostatic (growth inhibition) or even a bactericidal (antibacterial) influence. Therefore it has the ability to exert a bactericidal effect at minute concentration (Percival *et al.*, 2005) [14].

Aegle marmelos is extensively described in the Vedic literature for the treatment of various diseases like jaundice, constipation, dysentery, stomachache, acute bronchitis chronic diarrhea stomachic, fever, asthma, inflammations, febrile delirium, snakebite, abdominal discomfort, acidity, burning sensation, smallpox, epilepsy, indigestion, leporsy, myalgia, spermatorrhoea, leucoderma, eye disorders, ulcers, mental illnesses, nausea, sores, swelling, thirst, thyroid disorders, tumors, ulcers and upper respiratory tract infections (Sekar *et al.,* 2011) [18]. In the present study, we have demonstrated the prospect of using *A. marmelos* leaf extracts for the synthesis of AgNPs.

Materials and Methods

Preparation of plant extract

About 5 g of plant powder was weighed and ground by mortar and pestle. It was mixed with 25 ml of Ethanol. The ground solution was centrifuged at 8000 rpm for 5 mins. After centrifugation the supernatant was collected and used as reducing agent.

Biosynthesis of Silver Nanoparticles

1 mM (169 mg/l) of silver nitrate were weighed exactly and dissolved in sterile distilled water (100 ml) was freshly prepared and used for further studies. The 25 ml of centrifuged plant extract were taken separately. Add 225 ml of 1mM aqueous solution of Silver nitrate ($AgNO_3$) into extract for reduction of Ag+ ions separately and kept at room temperature for incubation. After addition of plant extract and silver nitrate solution the mixture were incubated in dark condition (to minimize the photo activation of silver nitrate) at room temperature.

Characteriation of Silver nanoparticles

The character studies of silver nanoparticle synthesised from the plant extract was done by UV- Vis spectral analysis, FT-IR analysis, SEM, DLS and XRD analysis.

UV-Visible spectrum

UV-Visible spectrum is one of the important techniques to ascertain the formation of metal nanoparticle synthesised. The aqueous silver nitrate solution (5 mM) was taken in a test tube and mixed with plant extract of centrifuged samples separately. The reduction of Ag^+ to Ag^- done by the plant extract was recorded by UV-Visible spectroscopy (Double beam spectrophotometer 2203) from 200 to 700mnm.

Fourier Transform Infra-Red Spectroscopy (FTIR)

The experimental procedure after incubation with 1 mM silver nitrate solution forms brown colour sediment. The sediment of the aqueous solution was centrifuged at 8000 rpm for 8 minutes and the resulting suspensions were dispersed in 10 ml sterile distilled water. The centrifugation and dispensing process were repeated 3 times. There after the purified sediments were air dried to obtain dried powder. FTIR for nanoparticles was done using FTIR spectrophotometer, Perkin Elmer make- Model spectrum one in the range from 4000 – 400 cm^{-1} at a resolution of 4 cm^{-1} was used. The dried experimental sample was mixed with KCl procured from sigma. This sample disc was prepared by pressing with disc preparing machine and placed in FTIR for analysis of nanoparticles.

SEM

Scanning electron microscopy was carried out using a JEOL-MODEL-6390 microscopy. The dried silver nanoparticles were freeze dried and their structure was analysed by SEM. Thin film of the sample was prepared on a carbon coated copper grid by just dropping a very small amount of sample on the sample grid, extra solution was removed and then the film on the SEM grid was allowed to dry putting it under a mercury lamp for 5 minutes. Each sample was analysed by the scanning electron microscope.

Dynamic Light Scattering

Dynamic light scattering (DLS) which is based on the laser diffraction method with multiple scattering techniques was employed to study the average particle size of silver nanoparticles. The prepared sample was dispersed in deionised water followed by ultrasonication. Then solution was filtered and centrifuged for 15 min.at 25º C with 5000 rpm and the supernatant was collected.

The supernatant was diluted for 4 to 5 times and then the particle distribution in liquid was studied in a computer controlled particle size analyzer (ZETA sizer Nanoseries, Malvern instrument Nano Zs).

X – Ray Diffraction (XRD)

The crystalline metallic silver was confirmed by XRD. The XRD measurement of bioreduced silver nanoparticles solution obtained was purified by repeated centrifugation at 10,000 rpm for 10 mins followed by redispersion of the pellet of silver nanoparticles in sterile distilled water. Further XRD instruments of the plant broths synthesized Ag nanoparticles solution were drop coated onto glass substrate and carried out on Shimadzu – MODEL XRD 6000 operated at a voltage of 40 Kv and a current of a 30 mA with Cu Kα radiation in a θ – 20 configurations.

Antibacterial activity

The antibacterial activity was tested by disc diffusion technique. The bacterial strains were procured from the culture collection in the MTCC (Microbial Type Culture Collection), Chhattisgarh. These include Gram positive organism: *Staphylococcus aureus* (MTCC 3160),) and *Streptococcus pyogenes* (MTCC 442) and Gram negative organism: *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424) and *Klebsiella pneumonia* ((MTCC 3384). Commercially available blank sterile discs (Hi Media Laboratories Pvt. Ltd, Bombay) of 6mm diameter were used 20 µl of various concentration (25, 50, 75 and 100 mg/ml) of plant extracts. The discs were used after drying them in an incubator at 37º C. Mueller Hinton agar plates were lawned with the inoculum using sterile cotton swabs dipped in the nutrient broth culture. Sterile disc impregnated with crude extract and the compound was allowed to diffusion for 5 minutes. The plates were then inverted for incubation at 37º C for 24 hrs. Examination of plate for inhibitory zone around each disc was done. The diameter of the inhibitory zone around each disc was measured. Also sterile discs impregnated with solvents alone were done as control. For each organism, triplicates were carried out and the average inhibition zone diameter was determined.

Results and discussion

Characterization of Nanoparticles

UV-visible analysis

`The colour change from brown to reddish brown was noted in 10 minutes. The formation and stability of AgNPs synthesized using *A. marmelos* extracts were initially examined by UV–Vis analysis, which is an important technique used to monitor metal mediated NPs in aqueous solution. The UV–Vis absorption spectra attained were typical and revealed the bioreductive formation of AgNPs; the plots are shown in Figure 1. A high-intensity surface plasmon resonance band is seen at 433nm, along with the synthesized AgNPs characteristic wavelength range.

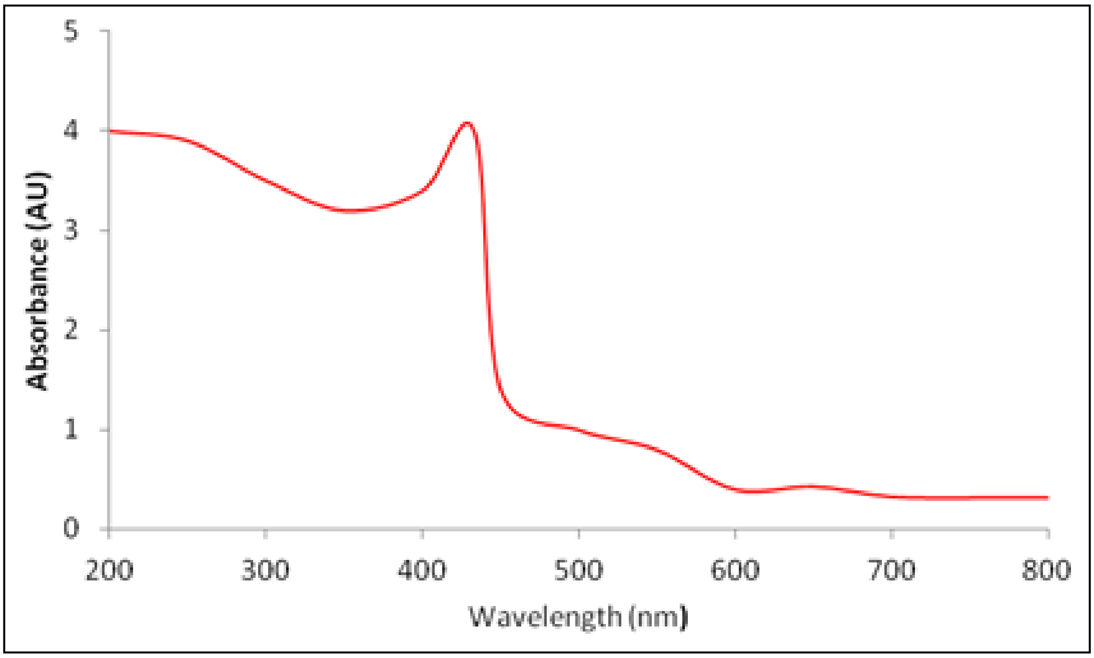

Fig 1: UV – visible Spectral analysis of nanoparticles synthesized from *Aegle marmelos*

FTIR analysis

The FTIR analysis of the synthesized silver nanoparticles exposed the two-fold function of the *A. marmelos* extract as a bioreductant whose biomolecules participate in the reduction of the Ag+ ions, and as a capping agent that stabilizes the bioreduced silver nanoparticles. The surface chemistry of the silver nanoparticles synthesized from *A. marmelos* is revealed by the appearance in the FTIR spectra of IR bands at 3451. 49, 2000.70, 1633.29, 1384.07, 1270.22, 1066.09 and 621.17 cm−1 and by the peaks which correspond to various groups (Figure 2).

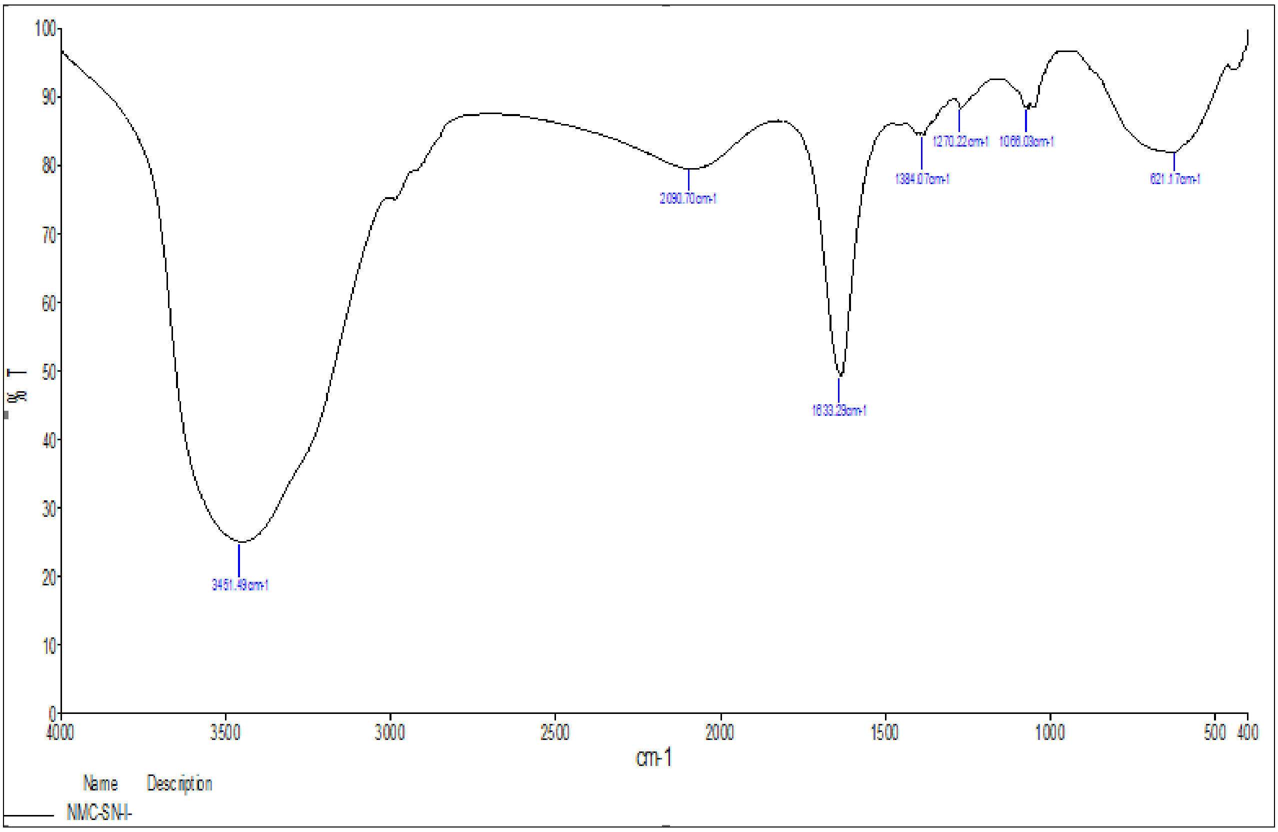

Fig 2: FTIR analysis of nanoparticles synthesised from *Aegle marmelos*

FE-SEM analysis

The 3D structure of silver nanoparticles has been elucidated using SEM image. The nanoparticles synthesized through *A. marmelos* were observed as spherical in nature as showed in Figure. 3. It was shown that relatively spherical and uniform AgNPs were formed with diameter of 73 to 75 nm. The SEM image of silver nanoparticles was due to interactions of hydrogen bond and electrostatic interactions between the bioorganic capping molecules bound to the AgNPs.

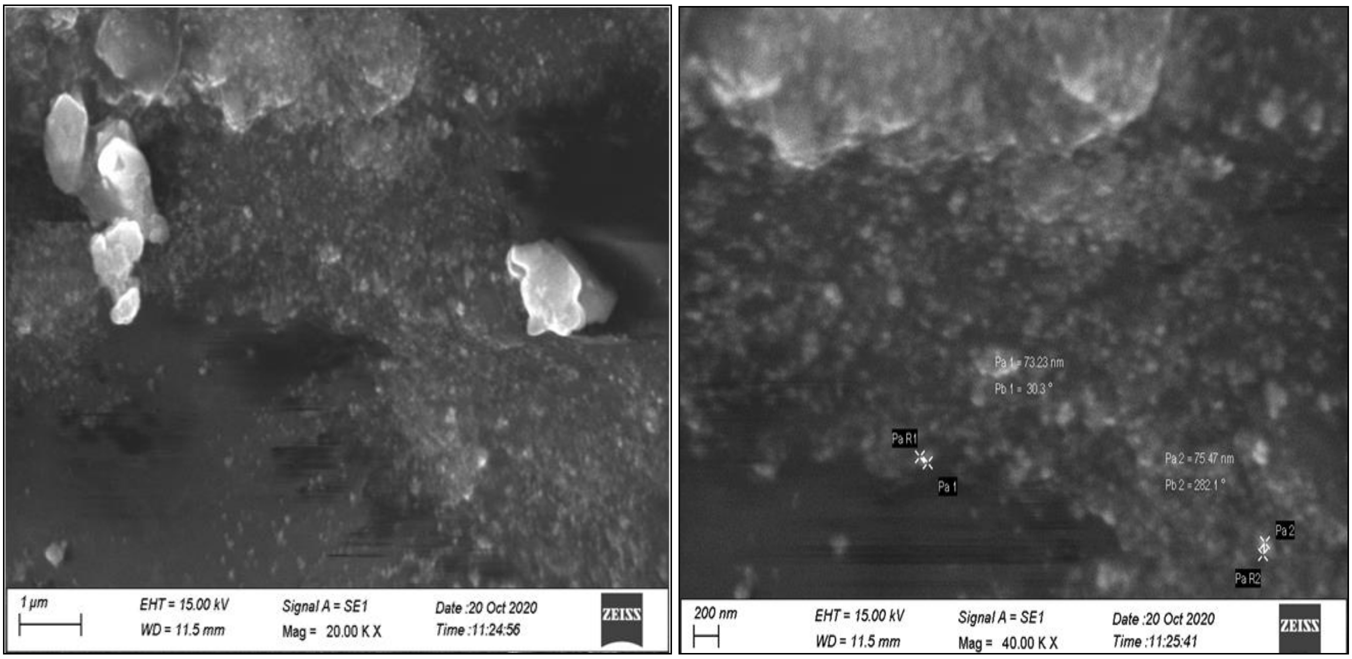

Fig 3: SEM analysis of nanoparticle synthesised from *Aegle marmelos*

DLS Analysis

The size of the AgNPs as analyzed by DLS analysis was 667 nm with PDI of 0.45 (Figure 4).

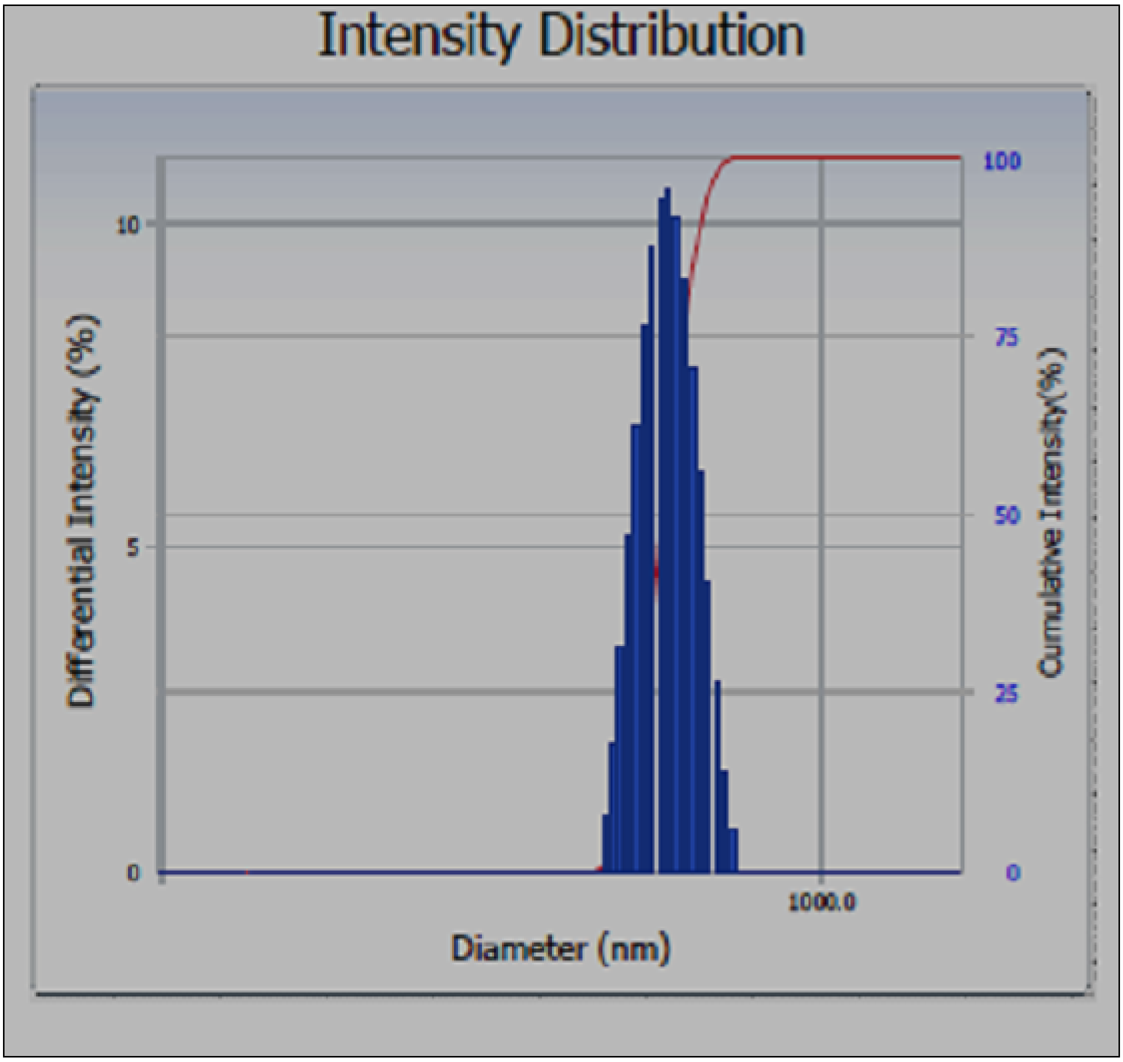

Fig 4: DLS analysis of nanoparticle synthesized from

Aegle marmelos

XRD analysis

XRD investigation was used to analyze the phase distribution, crystallinity and purity of the synthesized silver nanoparticle. The XRD pattern of the AgNPs synthesized through *A. marmelos* using ethanol extract was compared and interpreted using standard data. The major peaks at 27°, 32°, 38°, 46°, 54°, 57°, 67° and 76° (2θ values) which correspond to the Bragg's reflections from the (111), (200), (220) and (311) planes (Figure 5). Similar peaks and lattic were experiential by other researchers (Rajasekhar *et al*., 2012; Hussain *et al*., 2009) [15, 8]. It confirms the spherical phase of plant extract mediated the AgNPs. A comparison of our XRD spectrum with the Standard (JCPDS file no 04-0783) confirmed the formation of crystalline AgNPs, in accordance with the study of Kalimuthu *et al.*, 2008 [10].

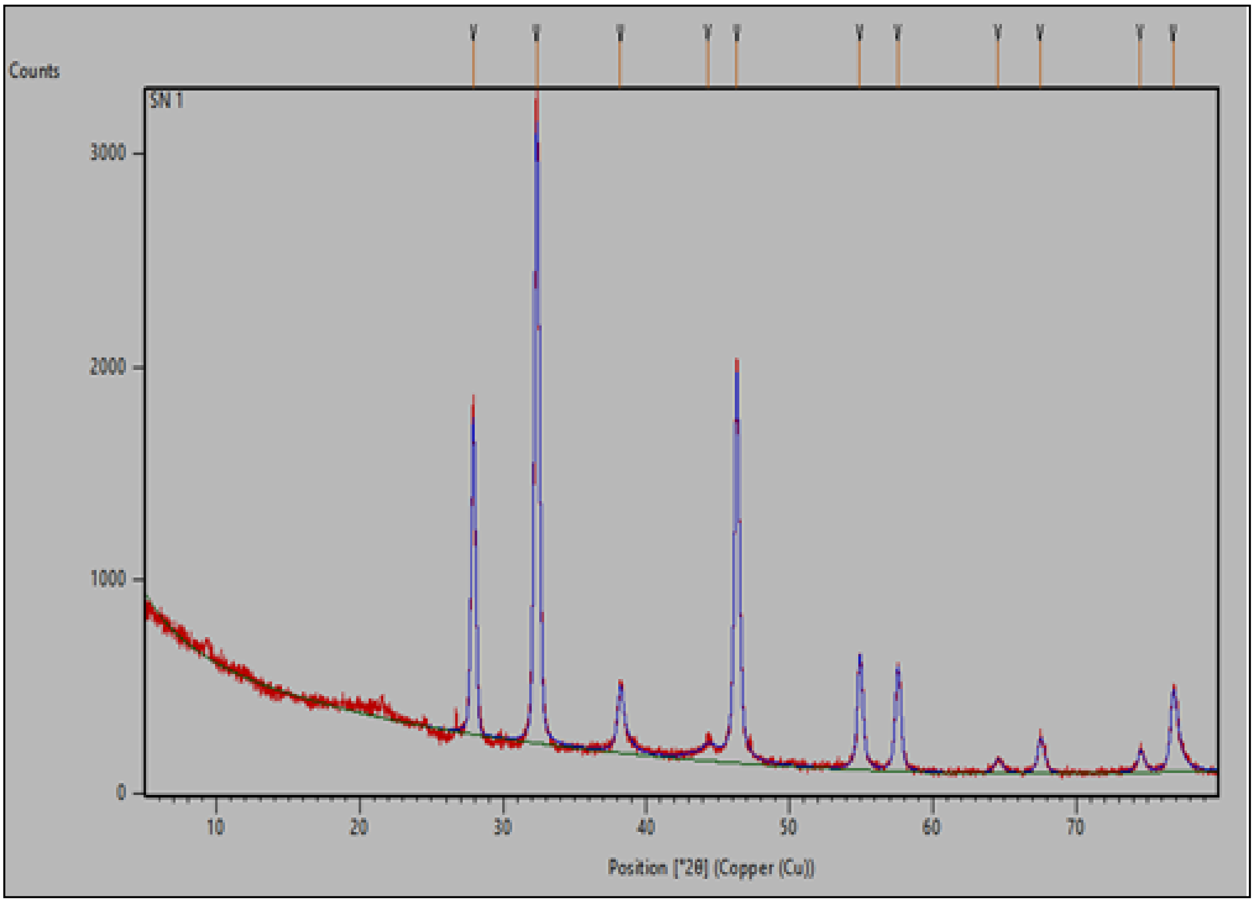

Fig 5: XRD pattern of nanoparticle synthesized from

Aegle marmelos

The average particle size was calculated by Debye-Scherer equation where full width at half maximum (FWHM) data was used (Singhal *et al.*, 2011) [19]. The average particle size was approximately about 6.43 nm. Debye-Scherer equation is $\tau=K\lambda/\beta\cos\theta$ where, τ is the particle size, K is a dimensionless shape factor, λ is the X-ray wavelength, β is the line broadening at half the maximum intensity (FWHM), θ is the Bragg angle (in degrees).

Antibacterial activity of Synthesized Silver Nanoparticle

In vitro antibacterial activity of the nanoparticles synthesized using ethanol extracts of *A. marmelos* was resolute by inhibition zones presented in Table 1 & Figure 6. Streptomycin used as a standard antibiotic at the concentration of 30µg/disc exhibited higher diameters of inhibition than other extracts. Totally the three extracts of different concentrations of selected plant extracts were tested for their bioactivity. Nanoparticles synthesized from ethanolic extracts showed substantial antimicrobial potential against test microbes than other organic solvent extracts. Most subject organism in the examination was *Staphylococcus aureus* against which the plant extracts showed higher activity determined by inhibition zone. Maximum antimicrobial activity were recorded against *Staphylococcus aureus* (IZ – 30.00 ± 0.13 mm) followed by *Klebseilla pneumoniae* showing (IZ – 26.00 ± 0.9 mm). The AgNPs synthesized using plant extract have been reported be highly toxic against gram positive as well as gram negative bacteria. Logaranjan *et al.*, 2012 have shown an enhanced antibacterial activity in AgNPs synthesized using *Ficus caricus* fruit extract and also with *V. tessellata* (Manjunath Hullikere *et al*., 2014) [12]. Similar results obtained even our study. The study has clearly established the antibacterial activity of AgNPs synthesized from ethanol extract was high than the normal extracted ethanol extract.

Table 1: Antibacterial activity of nanoparticle synthesized from *Aegle marmelos*

Test organisms	Inhibition zones in mm (diameter)			
	25µg/ml	50µg/ml	75µg/ml	100µg/ml
Staphylococcus aureus	15±0.06	17±0.22	23±0.32	30±0.13
Streptococcus pyogenes	10±0.14	13±0.01	15±0.39	21±0.67
Escherichia coli	10±0.19	13±0.7	17±0.57	22±0.4
Pseudomonas aeruginosa	12±0.29	16±0.31	17±0.37	24±0.43
Klebseilla pneumoniae	14±0.49	18±0.13	21±0.25	26±0.9

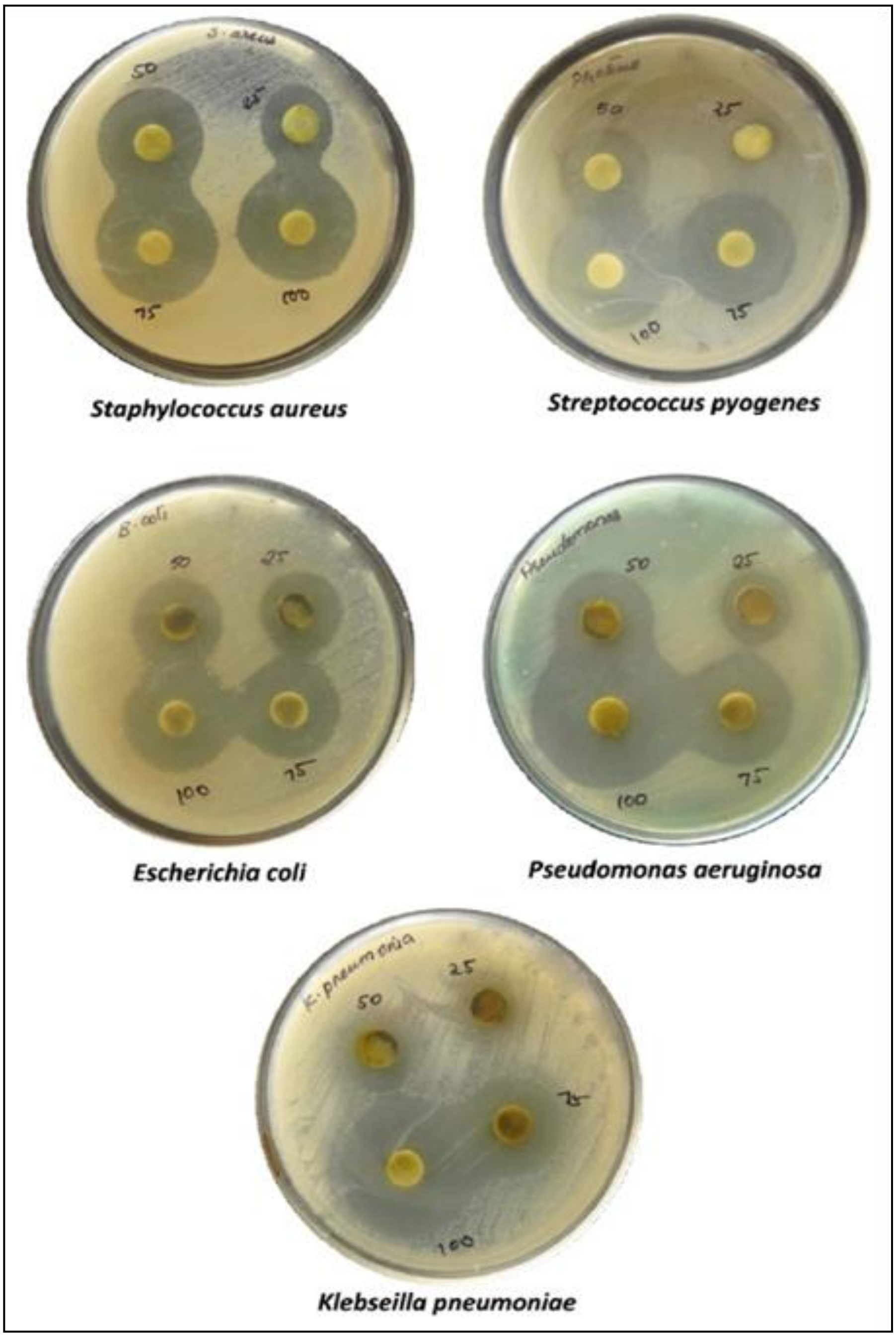

Fig 6

Conclusion

Silver nanoparticle was synthesized by adding 1ml of ethanol extract with 5ml 1mM concentration of silver nitrate solution and pH 6.2. The solution was allowed to incubate at room temperature for 45 minutes. Thus, the development of the brownish red colour established the silver nanoparticle synthesis. The solution was characterized using instrumentation analysis, thus the UV-Visible spectral examination exhibited a plasmon resonance band at 433nm and the FTIR analysis exposed the bioreductant molecules which facilitate the reduction of silver ions and as capping agent to stabilizes bioreduced silver nanoparticles. The XRD analysis established that the crystalline phase of nanoparticle synthesized. SEM analysis reveals the 3D structure of silver nanoparticle as spherical nature with diameter of 73 to 75 nm. The particle size of the nanoparticles was also studied using Dynamic light scattering system. The antibacterial activity of the nanoparticle showed highest record against *Staphylococcus aureus* (IZ – 30.00 ± 0.13 mm) followed by *Klebseilla pneumoniae* (IZ – 26.00 ± 0.9 mm).

Acknowledgement

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