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Green Synthesis of Silver Nanoparticles and Antimicrobial Studies in Aqueous Bulb Extract of *Eulophia graminea* Lindl.

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Abstract
Development of environmentally benign methods for synthesis of nanoparticles is an evolving important branch of nanotechnology. In the present study we report the green synthesis of stable silver nanoparticles (SNPs) from aqueous bulb extract of *Eulophia graminea*. These green synthesized nanoparticles are characterized by using UV-VIS spectroscopy, Zeta potential, FTIR, XRD, and TEM with EDAX. The colour change from grey to brown is observed upon synthesis and 430 nm peaks obtained from UV-VIS spectroscopic analysis. Zeta potential at -31.2 mV, negative value indicates the high stability of nanoparticles. FTIR spectroscopic studies confirms that phenols and proteins of bulb extract is main responsible for capping and stabilization of these SNPs. Crystallographic studies from XRD indicated the SNPs are crystalline in nature and owing 50 nm size. High resolution and magnification studies with TEM analysis revealed that the nanoparticles are spherical in shape having the size range from 5 to 50 nm. EDAX pattern of synthesized SNPs showed 62.53 weight percentage of Ag metal in the sample indicate the purity of sample. Further, the antimicrobial studies of these green synthesized SNPs show high toxicity towards different bacterial isolates. The results revealed that the selected medicinal plant possess the potentiality towards the synthesis of narrow range nanoparticles also combat with the pathogens.

Keywords
Eulophia graminea, Green synthesis, Silver nanoparticles, UV-Visible Spectroscopy and toxicity

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INTRODUCTION
The development of green processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology [1], [2]. The research on synthesized nano materials and their characterization is an emerging field of nanotechnology from the past two decades, due to their huge applications in the fields of physics, chemistry, biology and medicine [3]. Synthesis of silver nanoparticles has attracted considerable attention owing to their diverse properties like catalysis [4], magnetic and optical polarizability [4], electrical conductivity[5], antimicrobial activity [6] and surface enhanced Raman scattering (SERS) [7]. Biological methods of Nanoparticle synthesis using microorganisms [8]–[10], enzymes[11], fungus[12], and plants or plant extracts[13]–[17] have been suggested as possible ecofriendly alternatives to chemical and physical

methods. Sometimes the synthesis of nanoparticles using plants or parts of plants can prove advantageous over other biological processes by eliminating the elaborate processes of maintaining microbial cultures [18]. Further these biologically synthesized nanoparticles were found highly toxic against different multi-drug resistant human pathogens.

Genus Eulophia is highly diverse taxa occurs in a wide range of habitats and belongs to the family Orchidaceae. Eulophia, commonly known as corduroy orchids, [19]. Orchids in the genus Eulophia are mostly terrestrial herbs with either an underground rhizome or pseudo bulbs on the surface. The genus Eulophia comprises over 230 species, which are widespread from tropical; Southern Africa, Madagascar, from Neotropics to throughout tropical and subtropical parts of Asia and Australia. Only one species occurs in tropical America. In India, this genus is particularly distributed in tropical Himalaya and Deccan Peninsular region. Species Eulophia are used for several therapeutic purposes in different parts of India [20-21]. Crushed tubers and the extracted juice are used as vermifuge [22]. Bulb extract used as Ear drop.[23].

MATERIAL AND METHODS

Synthesis of SNPs

Eulophia graminea bulbs are collected from Jaapali area of Tirumala, Chittoor District of Andhra Pradesh, India. Species was identified and herbarium Specimen deposited With Voucher No.SR:01 in the Department of Biotechnology, Rayalaseema University, Kurnool. 5 gms of powdered bulb was extracted with 100 ml of milli *q* water on boiling water bath for 1 hour. Filter the content with whatman No. 1 filter paper and stored at room temperature for green synthesis of SNPs. 5 ml of plant extract was taken in 250 ml conical flask, titrated with 50 ml of 1mM Ag(NO3)2 at 60-80°C with the help of magnetic stirrer. The contents were centrifuged at 10000 rpm for 20 minutes to avoid the presence of any biological impurities. Further, it is used for characterization and antimicrobial studies.

Characterization of silver nanoparticles

UV–Vis absorption spectrum of SNPs was measured by using Nanodropp 800. Zeta potential analysed by HORIBA SZ-100, Fourier-Transform Infra Red (FT-IR) spectra of synthesised SNPs were analyzed in the range of 4,000 to 500 cm-1 with an IR AFFINITY-1, IR by ATR method. Crystalline nature of metallic silver nanoparticles was examined using an X-ray diffractometer (XRD) from Bracer, D8 advance, Germany. XRD-6000 equipped with Cu Ka radiation source using Ni as filter at a setting of 40 kV/30 mA.

Transmission electron microscopy (TEM) technique was used to visualize the morphology of the AgNps. The 200kV ultra-high-resolution transmission electron microscope (FEI-TECNAI G2 20 TWIN). TEM Grid were prepared by placing a 5 µL AgNp Solution on Carbon- Coated Copper grids and drying under lamp.

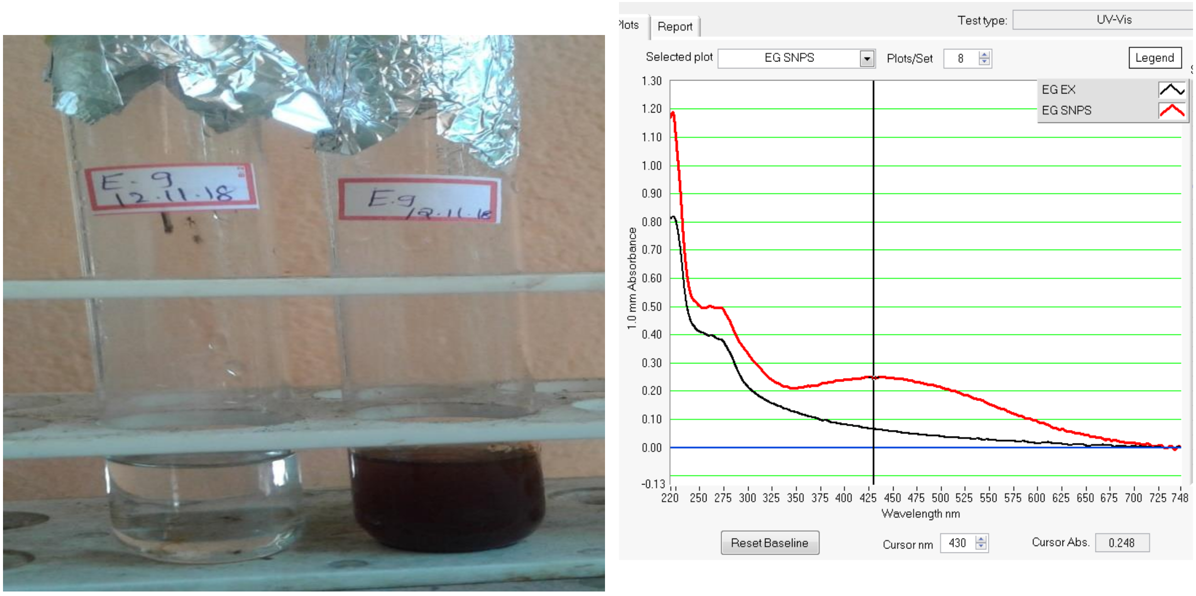
Antimicrobial studies of SNPs

The antimicrobial activity of green synthesized silver nanoparticles from bulb extract was analysed against two Gram positive bacterial strains like *Bacillus subtilis*, *Staphylococcus aureus* and two-gram negative bacterial strains like *Escherichia coli*, *Klebsiella pneumonia*. Disc diffusion method [24] was followed for testing antimicrobial activity against green synthesized SNPs and comparative studies were made with plant bulb extract as a positive control, 1mM Ag $(NO_3)_2$ as negative control and Streptomycin as the standard. Sterile discs of 7mm size were prepared from whatman No.1 filter paper and 20 µl of each extract was loaded on separate discs with the help of micro pipette and allowed to air dry for one hour in aseptic conditions. Freshly prepared nutrient agar media for bacterial culture substrate was poured into sterile petri plates and allowed 30 minutes for solidification. The plates were swabbed with microbial cultures and placed the previously prepared discs; the experiment was carried out in triplicates. The plates were incubated at 37 °C for 24 to 48 h then the zone of inhibition was measured.

RESULTS AND DISCUSSION

The formation of silver nanoparticles was monitored by UV-VIS absorption spectra. The colour change from grey to Dark Brown is observed after 60 minutes and a typical absorption peak obtained at 430 nm, it is due to surface plasmon resonance of silver nanoparticles in the reaction mixture (fig.1).As shown in figure 2, the SNPs obtained possessed a negative Zeta potential value. Zeta potential is an essential parameter for the characterization of stability in aqueous nanosuspensions. A minimum of -31.2 mV Zeta potential values is required for indication of stable nanosuspension[25].Zeta potential at-31.7mV, negative value indicates the high stability of Nanoparticles. So, this results clearly indicated that the particles are fairly stable due to the electrostatic repulsion. FTIR spectrum of synthesized SNPs was carried out to know the possible biomolecules responsible for capping and stabilization of nanoparticles. For this the FTIR spectrum was analysed between the scan ranges from 4000 to 500 cm^{-1}. Here the broad peaks obtained at 3311.78 cm^{-1} assigned for O—H (Stretch) bond of phenols, 1635.64 cm^{-1} assigned for N—H (Bend) bond of primary amines and 553 cm−1 assigned for C-Br (Stretch) of alkyl halides (fig.3). These FTIR studies suggested that the hydroxyl groups of phenols and amide groups of proteins forming a layer to the nanoparticles and acting as capping agents to prevent agglomeration and providing stability to the medium. The nature of the nanoparticles synthesized from leaf extract was analysed by X-ray diffraction analysis. The XRD Shows the derived SNPs with an intensive peak at 38.58 44.57 64.77 and 77.60 of 2θ degrees of X-axis corresponds to 111, 200, 220 and 311 Bragg Reflections of Y-axis (fig4). These Bragg reflections confirm that the nanoparticles are crystalline in nature.

UV-VIS ABSORPTION SPECTRA



(a) **(b)**

Fig.1 (a) Colour change grey to dark brown. (b) UV-VIS analysis of synthesized SNPs shows peak at 430 nm.

ZETA POTENTIAL ANALYSIS

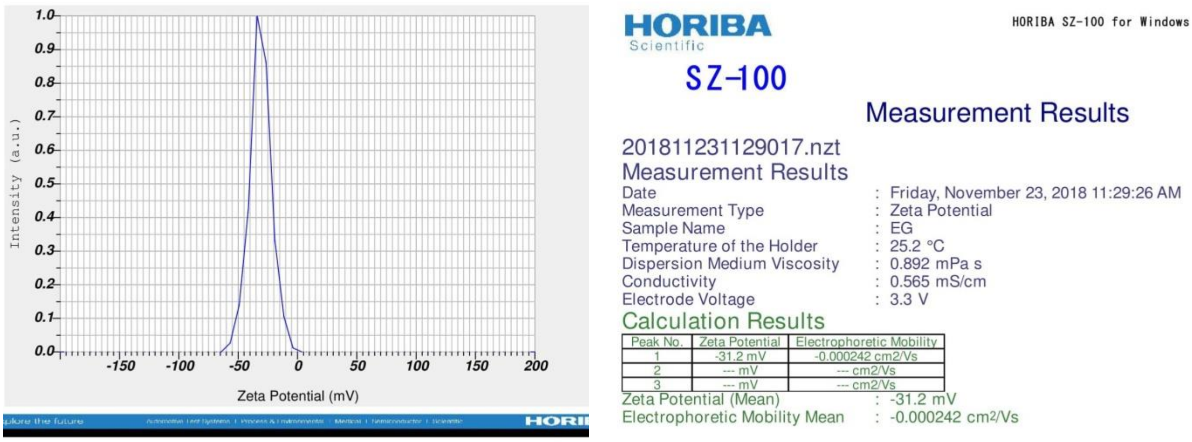


Fig.2 (a) Zeta potential of green synthesized SNPs from bulb extract of *Eulophia graminea*

FTIR SPECTRAUM

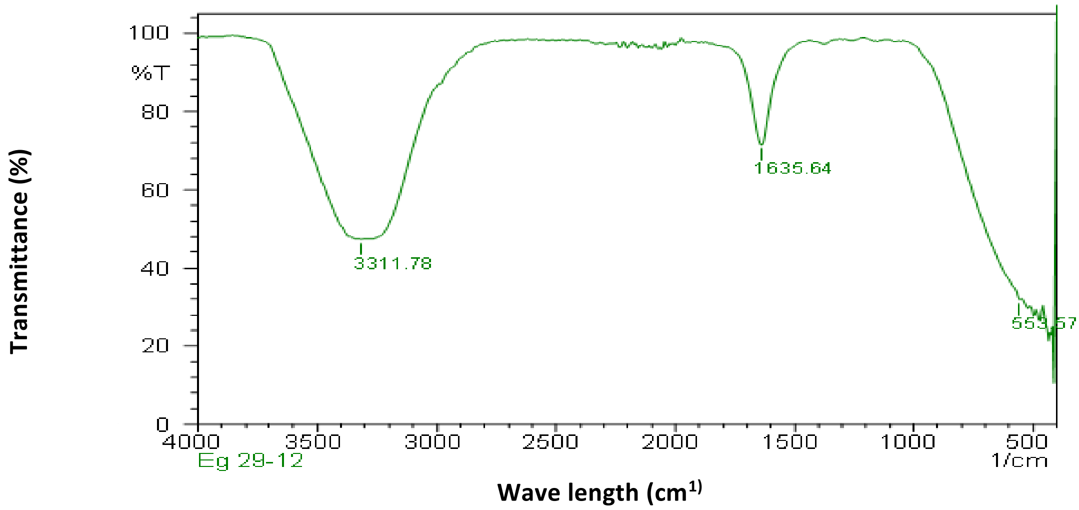


Fig.3 FTIR spectra of green synthesized SNPs from bulb extract of *Eulophia graminae* (3311 cm^{-1} assigned for O—H (Stretch) bond of phenols, 1635 cm–1 assigned for N—H (Bend) bond of primary amines and 553 cm^{-1} assigned for C-Br (Stretch) of alkyl halides.

X-RAY DIFFRACTION ANALYSIS

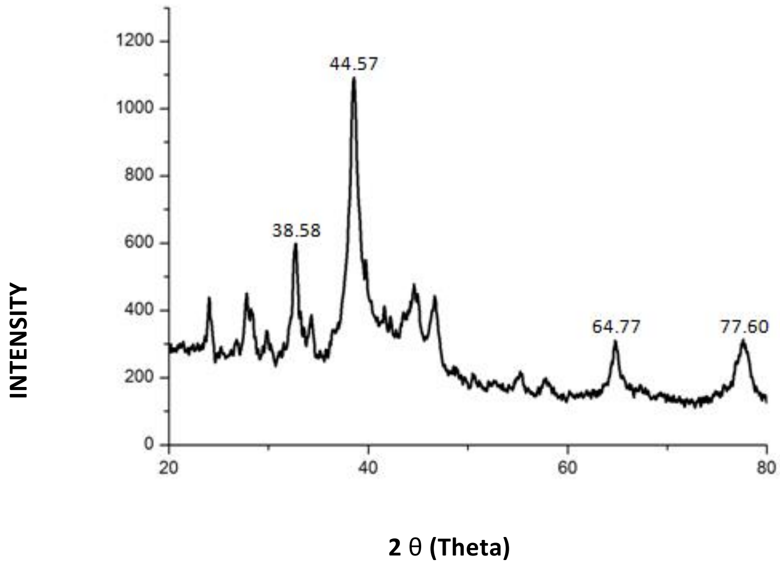
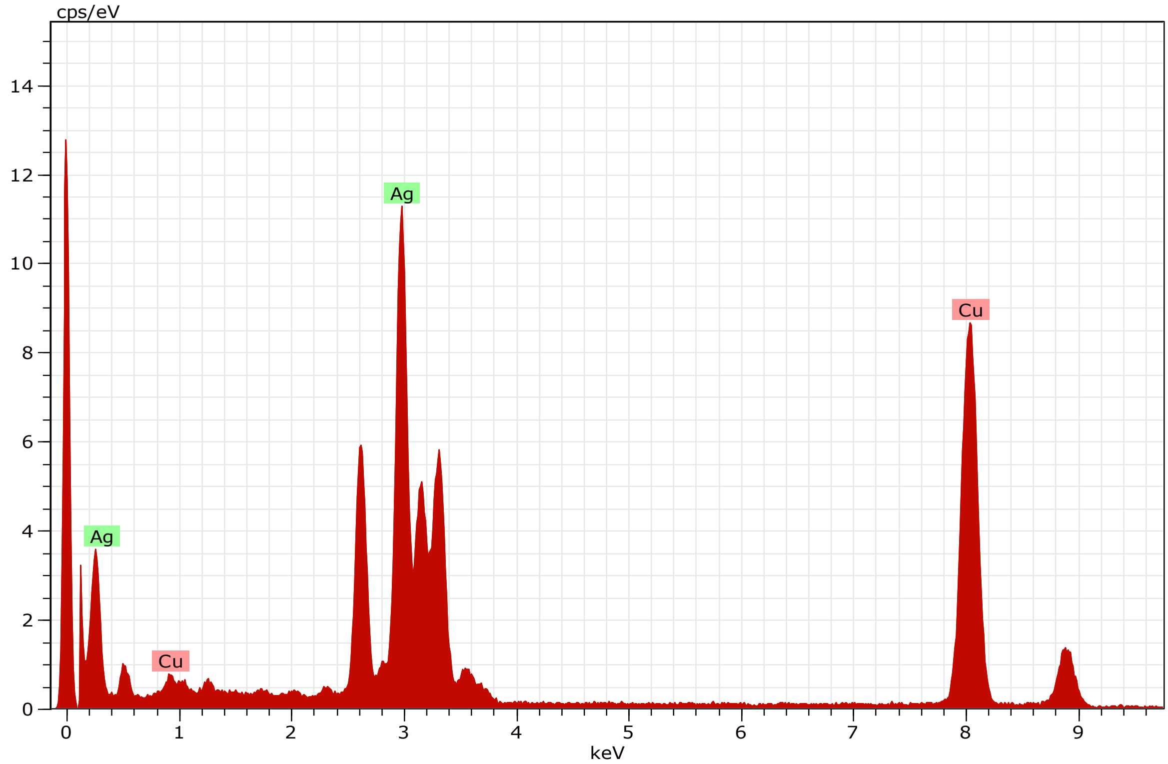


Fig.4 XRD pattern of green synthesized SNPs from bulb extract of *Eulophia graminea.* (Intensive peak at 38.58 44.57 64.77 and 77.60 of 2θ degrees of X-axis corresponds to 111, 200, 220 and 311 Bragg Reflections of Y-axis.)

TEM with EDAX analysis provides further insight into the morphology and size of the nanoparticles along with presence of different metal concentrations in the sample. 200 nm resolution studies of green synthesized SNPs show spherical shape with 20-50 nm size of nanoparticles. EDAX analysis was performed to know the percentage of Ag present in the sample. The EDAX spectra shows strong silver 62.53 % absorption peak along with different elements with their weight percentage like Copper

37.47 % (fig.5) and the results indicated that the reaction product has high purity of SNPs .Presence of C, N and O in the sample analyzed by EDAX indicates proteins as a capping material towards these silver nanoparticles [26]. Higher resolution studies with TEM analysis, to know the size, morphology and agglomeration pattern of nanoparticles. 50 nm resolution studies reveal the nanoparticles are 10-21 nm in size owing spherical shape without any agglomeration observed between the particles (fig. 6 and 7). From these microscopic studies with TEM analyses reveals these green synthesized silver nanoparticles from *Eulophia graminea* shows the size range from 5 to 50 nm. Having spherical shape without any agglomeration between the particles.

EDAX ANALYSIS



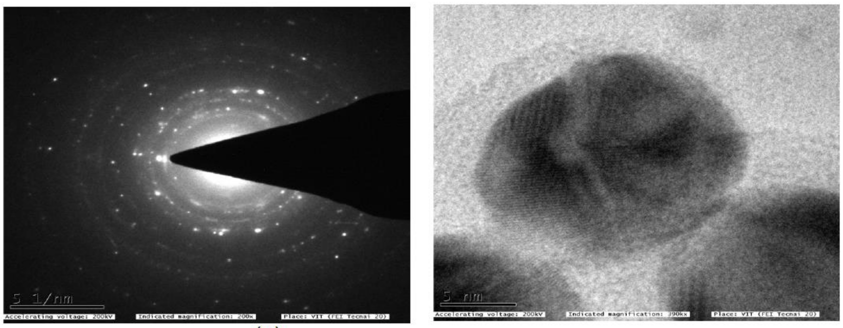
Spectrum: Spectrum 443-Eg

Element Series Net unn. C norm. C Atom. C Error (3 Sigma)
[wt.%] [wt.%] [at. %] [wt.%]

Element	Series	Net	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at. %]	Error (3 Sigma) [wt.%]
Copper	K-series	54662	37.47	37.47	50.43	3.49
Silver	L-series	76867	62.53	62.53	49.57	18.85
Total:			100.00	100.00	100.00	

Fig 5. EDAX analysis of green Synthesized SNPs shows 62.53 weight percentage.

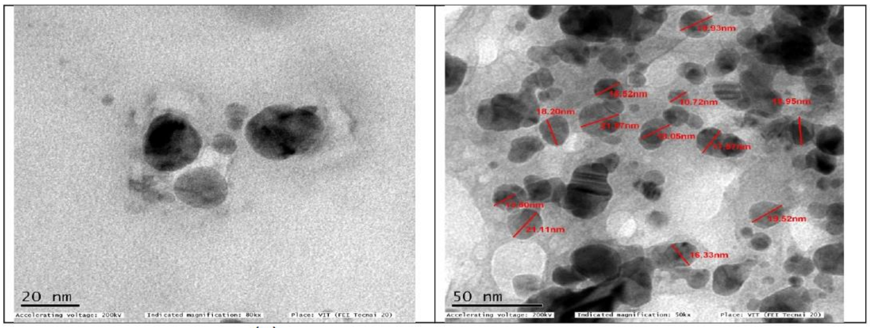
These green synthesized silver nanoparticles were assessed for antimicrobial activities against two gram positive and two-gram negative bacteria. Among these bacteria the highest inhibition zones was observed against *Klebsiella pneumonia*. (Fig 8, 9 and Table 1). The SNPs shows less significant effect on gram positive. Gram-negative bacteria are more susceptible when compared to the gram-positive bacteria.



(a)

(b)

Fig 6 (a) Selected area electron diffraction (SAED) of green synthesized SNPs, (b) 5 nm resolution studies of green synthesized SNPs



(c)

(d)

Fig 7 (c) 20 nm resolution studies of green synthesized SNPs shows mostly spherical shaped nanoparticles. (d) 50 nm resolution studies of green synthesized SNPs shows mostly spherical shaped nanoparticles with 10-21 nm size.

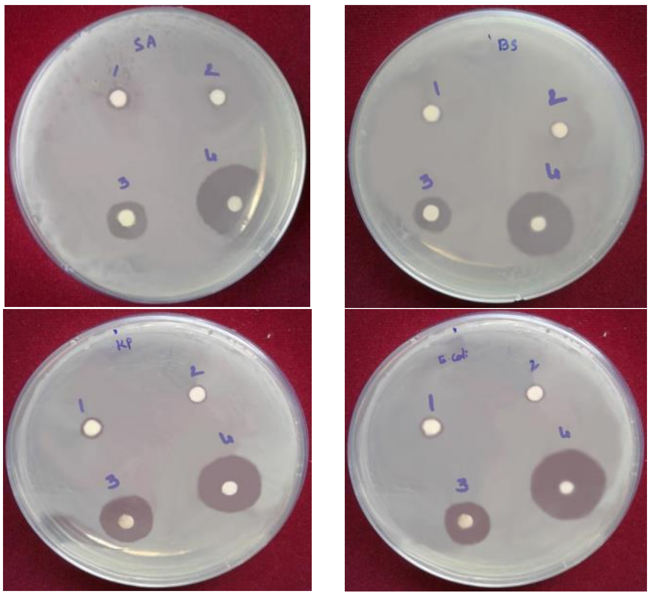


Fig.8 Antimicrobial activities of Synthesized SNPs from aqueous bulb extract of *Eulophia graminea*
(A) *S.aureus*, (B) *B.subtilis*, (C) *K.pneumonia*, (D) *E.coli*. (1) Plant extract (2) Ag (NO3)2
(3) SNPs (4) Streptomycin.

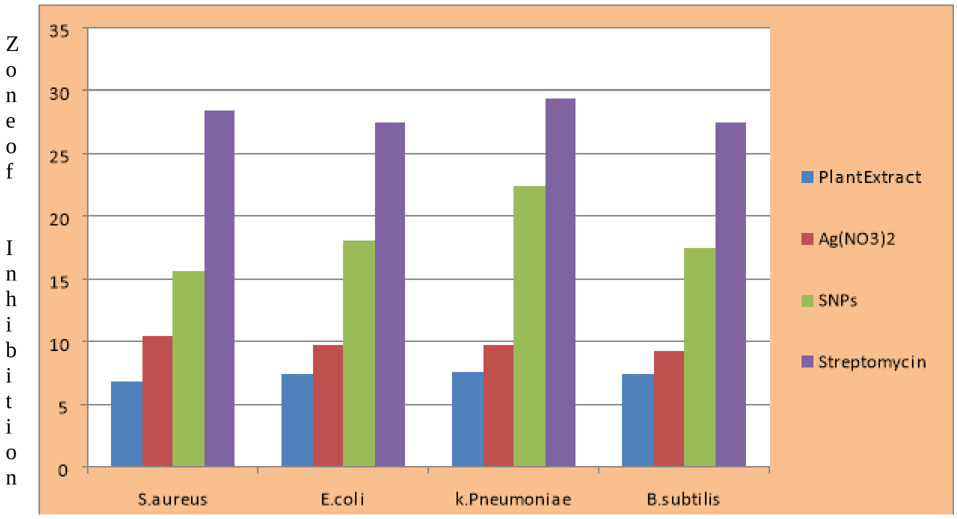


Fig 9 Zone of inhibition of different extracts on clinically isolated bacteria.

Table 1 Effect of different extracts and green synthesized silver nanoparticles on clinically isolated bacterial Strains.

Extracts 10mg/Disc	Diameter Zone of Inhibition in mm			
	S. aureus	***E. coli***	***K. pneumoniae***	***B. subtilis***
Plant Extract	6.8±0.32**	7.4±0.32**	7.6±0.32**	7.4±0.32**
Ag $(NO_3)_2$	10.5±0.32**	9.8±0.32**	9.8±0.32**	9.3±0.32**
SNPs	15.6±0.32**	18±0.57*	22.4±0.57*	17.4±0.32**
Control- Streptomycin	28.3±0.32	27.4±0.32	29±0.57	27.4±0.32

All the data are expressed as mean ±S EM: **p<0. 01, * p<0.05 as compared to Control group, n=3: (One –way ANOVA followed by Dunnett's test)

DISCUSSION

The gram-positive bacteria having thick layers of peptidoglycon (together with polypeptide proteins) when compared to the gram-negative bacteria and the penetration of SNPs through cell membrane is easy in case of gram-negative bacteria. 20-25 nm sized, spherical shaped silver nanoparticles were synthesized from *Olea europaea* leaf extract with good antibacterial activity Against *S. aureus*, *P. aeruginosa* and *E. coli* [27]. There are reports that the SNPs penetrate inside the bacteria and fungi causing damage by interacting with phosphorous and sulphur containing compounds such as DNA and proteins, resulting in cell death [28]

CONCLUSIONS

In the present study we develop eco-friendly and cost-effective method for silver nanoparticle synthesis in a greener way. This green method avoids deficiencies of chemical, physical and bacteria mediated approaches where plant extracts acts as reducing agents. Another advantage in this green synthesis approach is nanoparticles are stable for a long time. Further these nanoparticles showed good antimicrobial activity against clinically isolated pathogenic microorganisms. Hence, the production of of silver nanoparticles at lower cost with natural sources is an utmost important to industrial scale due to high importance in various medical fields. Quantity production of nanoparticles with little volume of plant extract is high measurably significant in this medicinal plant.

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