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Green Synthesis of Silver nanoparticles and Antioxidant, Antibacterial Studies in Fruit Aqueous Extract of *Pterolobium hexapetalum* (Roth.) Sant and Wagh.

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Abstract

The plant mediated synthesis of nanoparticles has Significant application in biomedicine due to its novel properties and its eco-friendly nature. The present study deals with the biosynthesis of stable silver nanoparticles (SNPs) from fruit aqueous extract of *Pterolobium hexapetalum.* The synthesized nanoparticles are characterized by UV–VIS spectroscopy, Zeta potential, FTIR, XRD, with TEM and EDAX. Colour change, observed from Gray to light brown indicates the formation of nanoparticles and UV–VIS surface plasmonresonance spectroscopy observed at 420 nm further confirmed the synthesized nanoparticles as SNPs. FTIR spectroscopic studies confirm that phenols and proteins of fruit extract is mainly responsible for capping and stabilization of synthesized SNPs. The XRD data shows crystalline nature of nanoparticles and EDAX measurements reveal the 42.75 % of Ag metal. Zeta potential at -23.7 mV, negitive value indicates the high stability of Nanoparticles. TEM microscopic analysis revealed that the size of synthesized SNPs ranging from 14.9 to 34.1 nm with spherical shape. Further, Antimicrobial studies of AgNPs showed highest Zone of Inhibition against *Staphylococcus aureus* (22.25mm) among bacterial strains. Antioxidant activity of AgNPs shows 71.67 %.

Keywords
Biomedicine, ecofreindly, stabilization, zone of inhibition, *Staphylococcus aureus,* Phenols.

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INTRODUCTION

Nanoparticles are gaining importance in the fields of biology, medicine [1]. Recent studies are focused towards synthesis of nanoparticles like, iron, copper, calcium, gold, palladium, zinc and silver using plant materials. Silver has been recognized [2]. Hence, among the metal nanoparticles, sliver nanoparticles (SNPs) synthesized from medicinal plants has received much attention in various biological activities like antibacterial [3] and antifungal [4]. The reducing agents involved in the synthesis include various water-soluble metabolites such as alkaloids, phenolic compounds, terpenoids, flavones, quinines, organic acids, polysaccharides, proteins, and co-enzymes which are available in the plant extract [5]. Biosynthesis of nanoparticles using plant materials is easy, efficient, and ecofriendly in comparison to chemical mediated or microbe mediated synthesis [6].

Pterolobium hexapetalum (Caesalpiniaceae) is a medicinal herb used (fig.1) by the Chenchu tribes of Nallamalai hills. Stem bark used for fever, cough, tooth ache, chest pain, dog bite (Rabies), vomits, heat boils; diahorrhoea, constipation and piles, bone fracture, jaundice, ulcer, skin infection, wound healing, flowers against venereal diseases, skin infection [7]; fruit and seeds cure diarrhea, constipation, piles, cough and cold , treating ulcer

[8]; leaves against delivery pains [9,10]. Stem bark decoction in case of whooping cough of infants and bark extraction dyspepsia in cattle [11]. *P.hexapetalum* is a characteristic dry deciduous straggling shrub on forest tree canopy with mass flowering known as "Bhoca" in the Nilgiris "Yerrachiki" in telugu, commonly known as Indian red wing. It is also a major source of nectar and pollen for honeybees which yield very sweet, pleasant aroma honey [12]. *P.hexapetalum* leaf and stem bark alcohol , methanol, ethyl acetate , benzene and chloroform extracts reveals the presence of high quantities of alkaloids, flavonoids, phenols , glycosides, tannins, quinines and steroids. There is no reports of lignins, saponins and fixed oils. Effective antibacterial activity was observed on four selected bacterial strains with leaf and stem bark hot water and methanol extracts at 10mg/disc. MIC values on *Staphylococcus aureus* and *Bacillus subtilis* 0.312 mg. *Pseudomonas aeruginosa* 0.625 mg and *Escherichia coli* 1.25 mg [13].

MATERIAL AND METHODS

Medicinal plant material collection and Identification

Pterolobium hexapetalum was collected from Tirumala Forest, during the months of July and December. The plant was authenticated by Prof. N. Yasodamma and voucher specimens BS 01, BS 02 were prepared as per the standard method [14] and deposited in the herbarium, Department of Botany.

Synthesis of *P.hexapetalum* Frit SNPs

5 gms fruit dry Powder was used for the extraction 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, antioxidant and antimicrobial studies. [15].

Characterizations of *P.hexapetalum* Fruit extract SNPs

UV–Vis absorption spectrum of SNPs was measured by using Nanodrop 800. Zeta potential analysed by HORIBA SZ-100, Fourier-Transform Infra-Red (FT-IR) spectra of synthesized SNPs were analyzed in the range between 4,000 to 500 cm-1 with an IRAFFINITY-1,IR by ATR method. Crystalline nature of metallic silver nanoparticles was examined using an X-ray diffractometer (XRD) from Bruker, 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 200 kV 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. [16-21].

Antioxidant Activity [DPPH]:

DPPH (2,2-diphenyl-1-picryl hydrazyl) free radical scavenging method involves the stock solution prepared by dissolving 4 mg of DPPH in 100 ml of methanol and stored at 20 °C 2 ml of this solution was added to 1 ml of methanol solution containing test samples of *P.hexapetalum* aqueous fruit extract and *P.hexapetalum* fruit AgNPs at different concentrations (50-250 µg/ml). Ascorbic acid was used as a standard. RSA (%) = [*(Ac-As)/Ac*] × 100 Where RSA is Radical scavenging activity, *Ac* is the absorbance of the control, and *As* is the absorbance of the sample or standard [22].

Antimicrobial studies of *P.hexapetalum* fruit extract SNPs

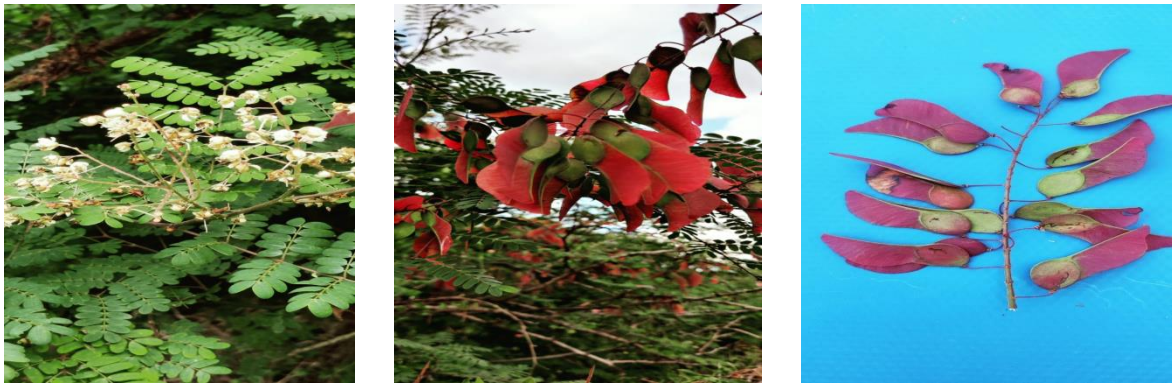
The antimicrobial activity of green synthesized silver nanoparticles of *P.hexapetalum* Fruit extract was analyzed against two Gram positive bacterial strains like *Bacillus subtilis* (MTCC441), *Staphylococcus aureus* (MTCC731) *and* Two Gram negative bacterial strains like *Escherichia coli*,(MTCC443) and *Klebsiella pneumonia*(MTCC741) using Disc diffusion method [23]. Comparative studies were made with Fruit extract as a positive control, 1mM Ag (NO3)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 under aseptic conditions. Freshly prepared nutrient agar medium substrate for bacterial culture 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 hrs then the diameter zone of inhibition was measured.

RESULTS

Collection, description of selected medicinal plant

Pterolobium hexapetalum was collected from Tirumala Forest. *Pterolobium hexapetalum* is a straggling prickly shrub, thorns recurved. Leave 2-pinnnate, leaflets oblong-oblanceoalte, entire obtuse, flowers white, in axillary or terminal racemes. Sepals and petals 5 each. Stamens 10ovary

sessile, ovule1, style subulate, stigma dilated. pods samaroid, oblong winged reddish [24].



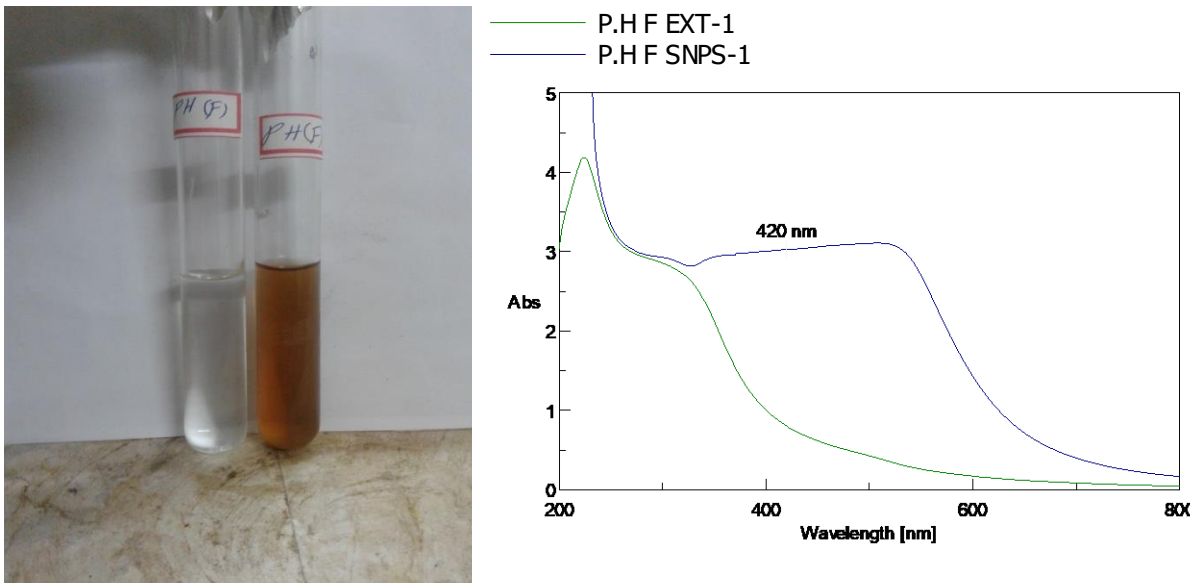
Habit Fruits

Fig.1 *Pterolobium hexapetalum*

Synthesis and characterization of SNPs.

UV–visible spectral analysis:

The formation of *P.hexapetalum* Fruit extract Silver Nanoparticles was monitored by UV-VIS absorption spectra. The colour change from Grey to light Brown is observed and a typical absorption peak obtained at 420 nm, it is due to surface Plasmon resonance of silver nanoparticles in the reaction Mixture (fig.2 a,b).



(a) (b)

Fig.2 (a) UV-VIS analysis of *P.hexapetalum* synthesized SNPs shows peak at 420 nm. (b) Colour change grey to light brown.

Fourier Transform infra-Red (FTIR) analysis:

The FTIR spectrum was analyzed between the scan ranges from 4000 to 500.The FTIR results at 3211.48 cm^{-1} assigned for O-H (Stretch) bond of phenols; 2937.59 cm^{-1} for N-H (Stretch) of Carboxylic acid; 2742.78 cm^{-1} for C-H (Stretch) alkynes ; 1689.64 cm^{-1} for C=C(Stretch) Ketones; 1602.85 cm^{-1} for C-C (Stretch) Aromatics; 1444.83 $cm^{-1;}$ for C-H (Stretch) Alkanes; 1330.88 cm^{-1} for C-N (Stretch) aromatic amines ; 1203.58cm^{-1} for C-N (Stretch) aliphatic amines. 1068.56 $cm^{-1;}$ for C-H (Bend) alkanes; 866.04 cm^{-1} for O-H 'oop' aromatic; 761.68 cm^{-1}; for C-Cl (Stretch) Alkyl halides; 594.08 & 459.06 cm^{-1} for C-Br (Stretch) Alkyl halides. (fig.3).

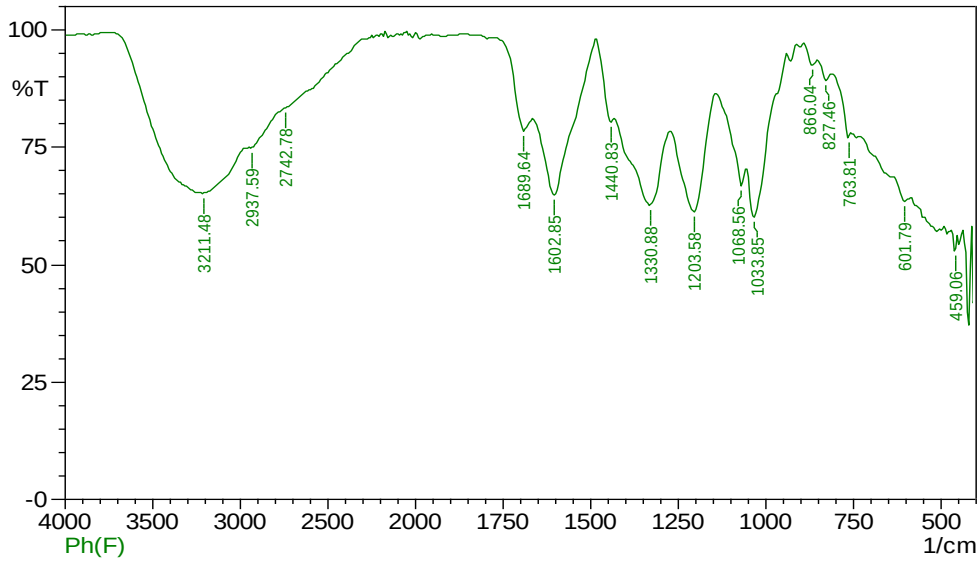
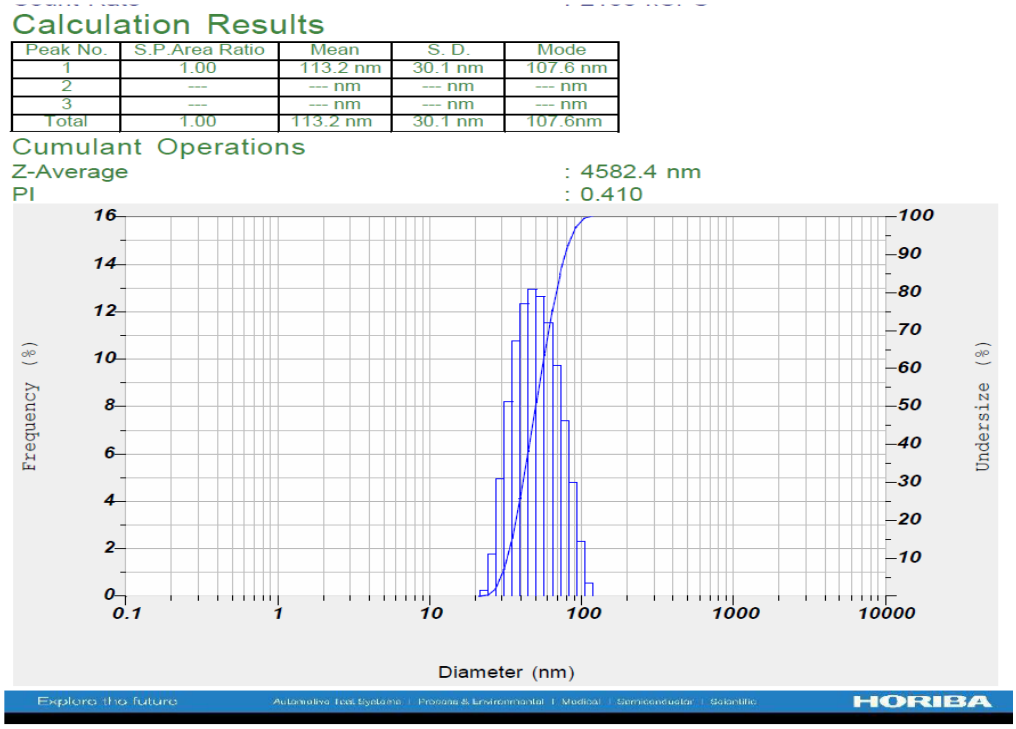


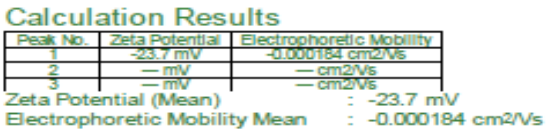
Fig.3 FTIR spectra of SNPs from Fruit extract of *Pterolobium hexapetalum.*

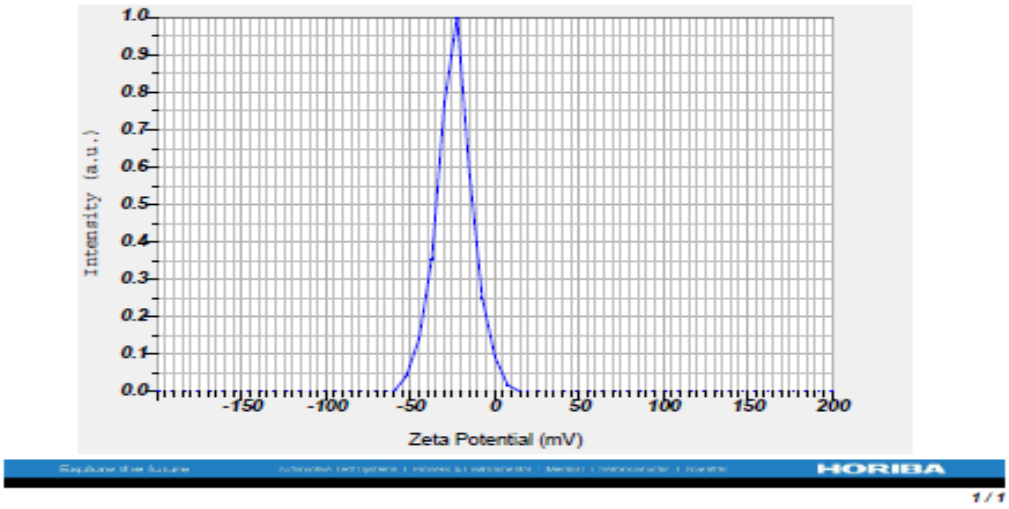
Particle size and Zeta potential analysis:

The particle size of the biosynthesized AgNPs is detected by the intensity and laser diffraction method using the biosynthesized colloidal solution in which the AgNPs are polydispersed in mixture solution. The distribution of AgNPs are in the range of 10nm to 100 nm in size with and the average size of synthesized AgNPs was found to be 113.2 nm (Fig. 4 a &b) with and PI value of 0.410 (poly disperse index). Further the zeta potential analysis of AgNPs was detected to be -23.7 mV, due to its high negative zeta potential it prevent the AgNPs from agglomeration in the medium, leading to long term stability, because of the electrostatic repulsive force between the AgNps.



(a)





(b)

Fig.4: (a) Particle size (b) Zeta potential of SNPs from Fruit aqueous extract of *Pterolobium hexapetalum.*

XRD Analysis:

The nature of the nanoparticles synthesized from bark extract was analysed by X-ray diffraction analysis. The XRD Shows peaks for plant derived SNPs of *P.hexapetalum*. An intensive peak at 28.77; 29.99; 36.57; 40.06; 40.88; 43.70; 48.01; 49.08; 66.70 and 73.97 of 2θ degrees of X-axis corresponds to 400, 121,220, 301, 213, 114,321,303, 033,223, and 134 Bragg Reflections of Y-axis (JCPDDS No: 84-110861,841261) (fig5). These Bragg reflections confirm that the nanoparticles are crystalline in nature.

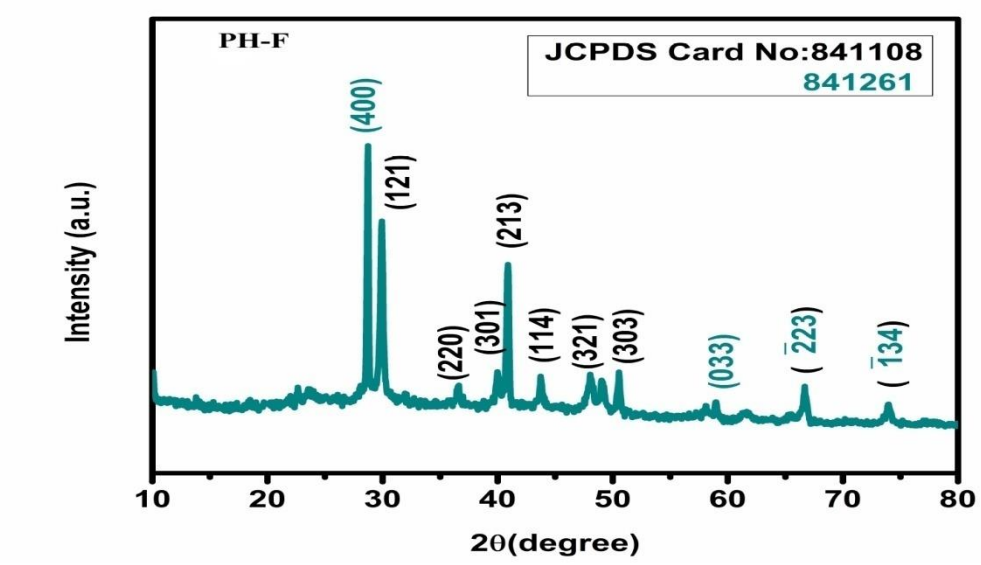


Fig 5 XRD pattern of SNPs from fruit extract of *Pterolobium hexapetalum*.

Transmission Electron Microscopy (TEM) and Energy Dispersive X-ray (EDAX) Analysis:

TEM with EDAX analysis (fig 6&7) provides further insight into the morphology and size of the nanoparticles along with presence of different metal concentrations in the sample. EDAX analysis of *P.hexapetalum* SNPs was performed to know the percentage of Ag present in the sample. The EDAX spectra shows silver 42.75 % absorption peak along with different elements with their weight percentage Carbon (43.69%), Copper (12.05%), and Oxygen (2.68%) and the results indicated that the reaction product has high purity of SNPs. Higher resolution studies with TEM analysis, to know the size, morphology and agglomeration pattern of nanoparticles.100 nm resolution studies of nanoparticles on TEM analysis reveals the nanoparticles are 14.9-34.1 nm in size owing spherical shape without any agglomeration observed between the particles

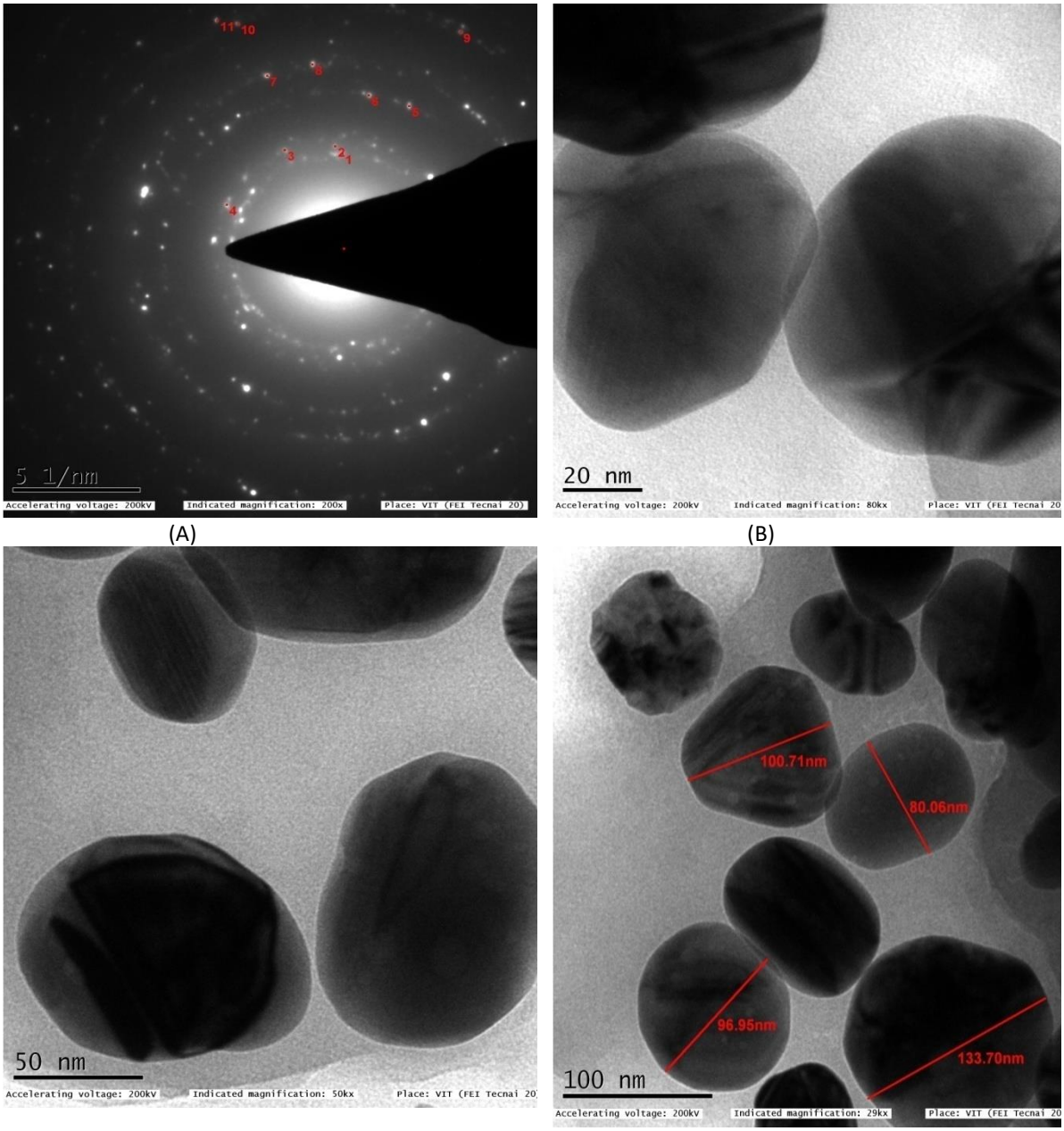
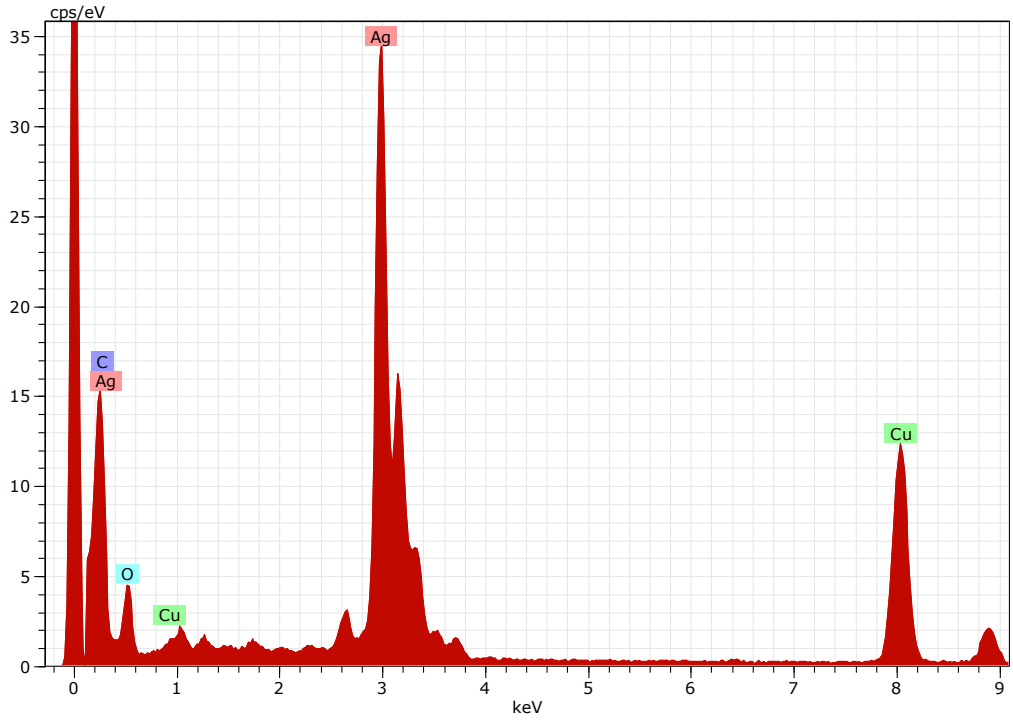


Fig 6 (A) Selected area electron diffraction (SAED) of *P.hexapetalum* fruit extract green synthesized SNPs, (B) 20 nm resolution SNPs. (C) 50 nm resolution SNPs. (D) 100 nm resolution nanoparticles with 14.9 -34.1 nm shows mostly spherical shaped nanoparticles.



Spectrum: Spectrum 1496 Ph-F

Element	Series	Net unn. C	norm. C [wt.%]	Atom. C [at.%]	Error (3 Sigma) [wt.%]
Silver	K-series	8964	42.75	42.75	9.18 4.20
Copper	K-series	29732	12.05	12.05	4.39 1.18
Carbon	K-series	13732	43.69	43.69	84.25 4.16
Oxygen	K-series	3315	1.51	1.51	2.18 0.23
Total:			100.00	100.00	100.00

Fig 7 EDS analysis of green Synthesized **AgNPs** of ***P.hexapetalum***

DPPH Antioxidant Analysis of *P.hexapetalum* SNPs

The aqueous fruit extract and synthesized AgNPs of *P.hexapetalum* showed better antioxidant potential when compare to standard ascorbic acid by DPPH scavenging assay method. Different concentrations ranging from 50-250 µg/ml of *P.hexapetalum* fruit aqueous extract and *P.hexapetalum* AgNPs; Ascorbic acid was taken as a positive control to compare the percentage activity of the aqueous fruit extract and silver nanoparticles. The antioxidant activity was increased in dose-dependent manner. The highest percentage activity was exhibited at 250 µg/ml *P.hexapetalum* fruit extract (60.33%) *P.hexapetalum* fruit AgNPs (71.67%) < Ascorbic acid (75.00%).From the results, it is concluded that silver nanoparticles of fruit extract possess good DPPH activity when compared to that of fruit extract alone (Fig.8 & Table.1)

Table 1: Percentage of DPPH Antioxidant Activity of *P.hexapetalum* SNPs

Concentration (µg/ml)	Aqueous Extracts	AgNPs	Ascorbic Acid
50 µg/ml	25.33 ± 0.33	39 ± 0.58	45 ± 0.58
100 µg/ml	36.33 ± 0.33	50.33 ± 0.88	57.33 ± 0.67
150 µg/ml	52.33 ± 0.67	65.33 ± 0.33	60.67 ± 0.33
200 µg/ml	58.33 ± 0.33	68.33 ± 0.33	68.67 ± 0.33
250 µg/ml	60.33 ± 0.88	71.67 ± 0.33	75 ± 0.58

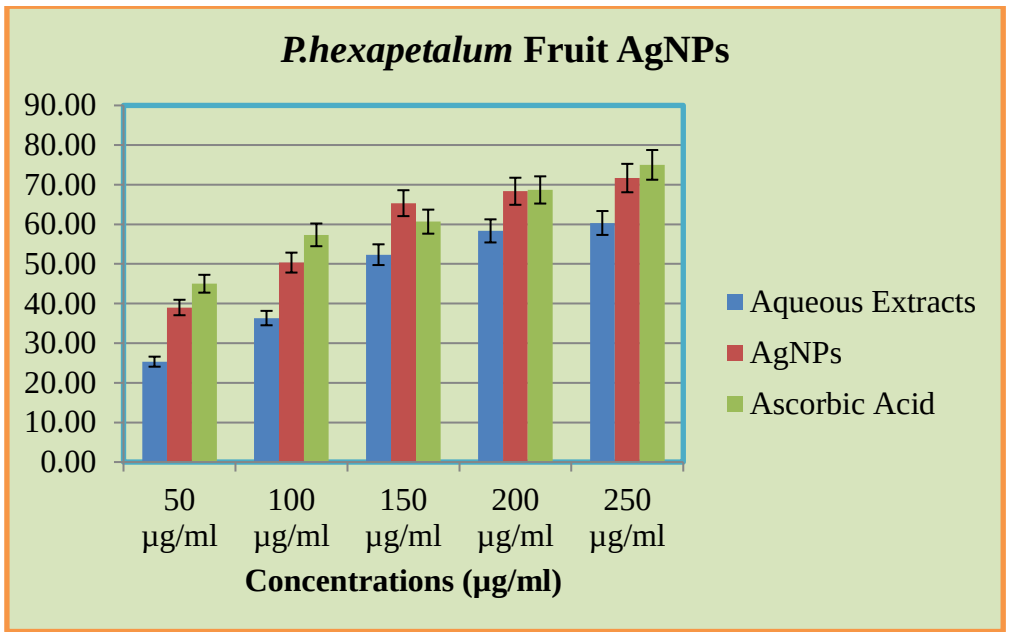


Fig 8: DPPH Radical Scavenging activity of *P.hexapetalum* SNPs

Antimicrobial activity of *P.hexapetalum* fruit AgNPs:

Green synthesized silver nanoparticles were assessed for antimicrobial activity against two gram positive and Two-gram negative bacterial strains. Among the bacteria the highest inhibition zone was observed in *Staphylococcus aureus* (22.25mm) followed by *Klebsiella pneumonia (*17.00 mm) (Fig 9, 10 and Table 2).

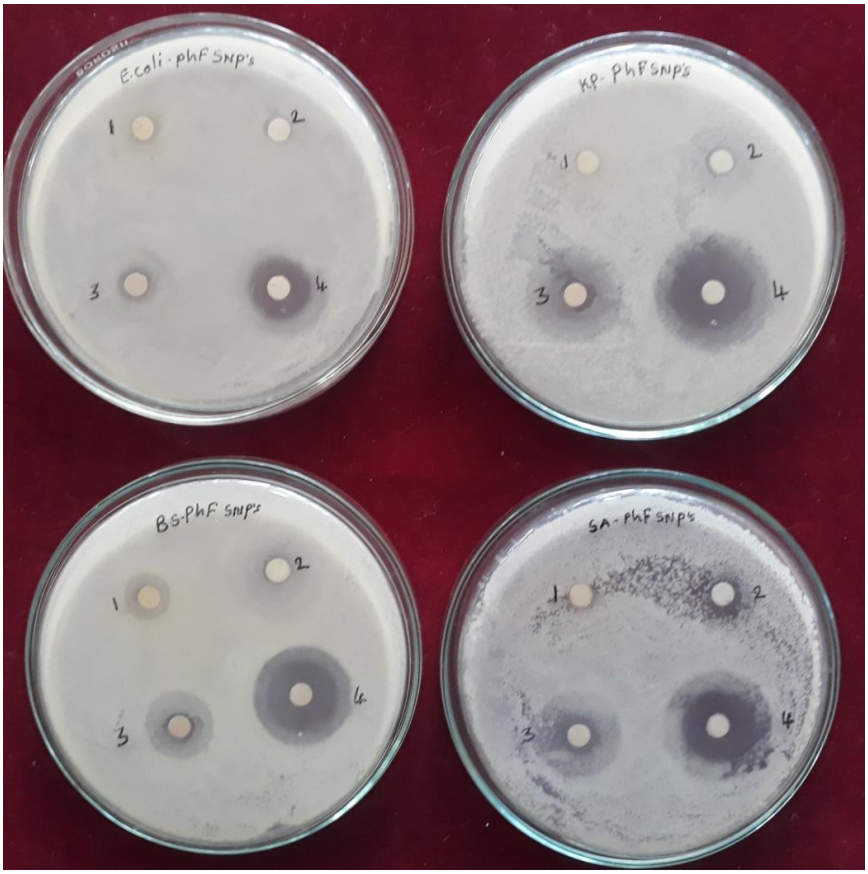


Fig.9 Antimicrobial activity of SNPs from fruit extract of *Pterolobium hexapetalum*
E.coli K.pneumonia, B.subtilis. S.aureus (1) Ag (NO3) (2) Plant extract (3) SNPs (4) Streptomycin.

Table 2 Antibacterial Effect of different extracts and silver nanoparticles of *P.hexapetalum* SNPs

	Zone if Inhibition (mm)			
Organism	**Plant Extracts**	**$Ag(NO_3)_2$**	**SNPs**	**Streptomycin**
E.Coli	6.75 ± 0.25	7.25 ± 0.48	9.25 ± 0.25	10.75 ± 0.48
KP	6.25 ± 0.25	7.5 ± 0.29	16.5 ± 0.29	22 ± 0.41
BS	11 ± 0.41	6.75 ± 025	15.75 ± 0.25	18.75 ± 0.48
SA	6.75 ± 0.25	8.25 ± 0.25	22.25 ± 0.48	23 ± 0.58

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)

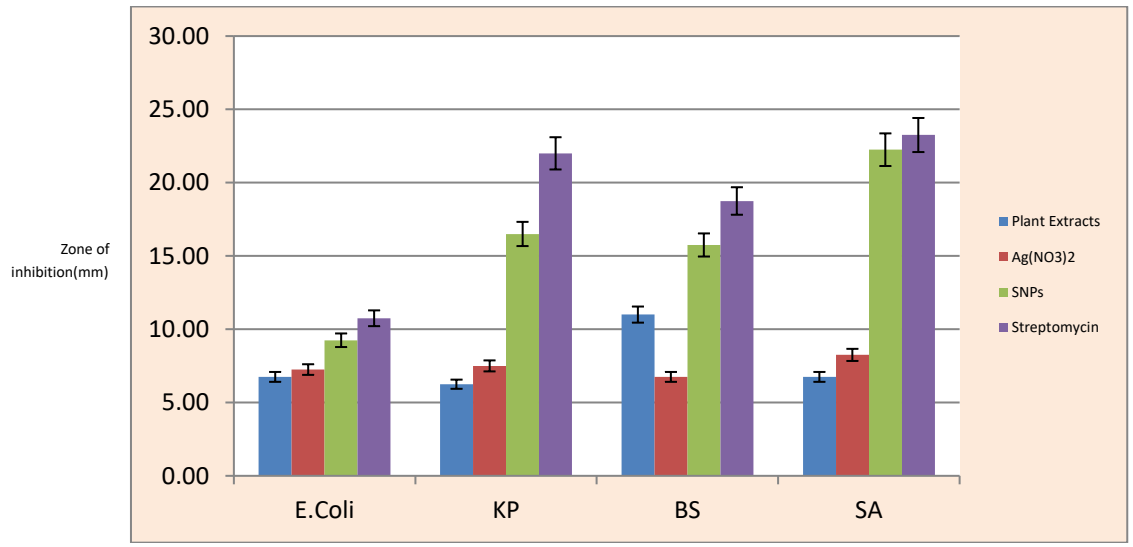


Fig 10 Zone of inhibition of *P.hexapetalum* different extracts on clinically isolated bacteria

DISCUSSION

Antibacterial activity of leaf *P.hexapetalum* on *B.subtilis*, *S.aureus, P.aeruginosa* and *E.coli* at 10 mg /ml with hot water and methanol extracts proved more effective twice to that of the control drug Gentamycin at 10mg /ml followed by stem bark extracts equal to that of the control drug, as the diameter zone of inhibition 21 to 22mm against all selected bacterial strains with leaf extracts; 13 to 15mm with stem bark extracts: 13-20 mm with drug control Gentamycin. MIC concentration ranges from 0.312 to 1.25 mg compared to Gentamycin 10mg against all bacterial strains [13]. Antifungal activity of *P.hexapetalum* leaf, fruit, flower, and stem bark aqueous, methanol and benzene extracts at 10mg/ml proved their effective inhibition on *A.niger* and *C.albicans* ranging with 30-35 mm zone of inhibition and MIC ranging from 0.156 to 0.625mg compared to that of control drug Nystatin nearly double the activity. Antibacterial and anti-fungal activities of *P.hexapetalum* were compared with earlier reports of the Caesalpiniaceae members like *Bauhinia*, *Cassia*, *Caesalpinia*, *Hardwickia*, *Peltophorum* and *Tamarindus*, *P.hexapetalum* antifungal activity at 10mg/ml was supported to that of the *Bauhinia purpurea* and *B.rufescens* bark methanol extracts at 100-150 mg/ml on *C.albicans* nearly with equal zone of inhibition and also 12-20mg/ml of MIC values [25& 26]. *B. tomentosa* flower ethanol extracts at 5mg/ml on *A.niger* and *C.albicans* showed equally effective activity to that of *P.hexapetalum* flower extracts , MIC values ranges from 0.312 to 1.25 [27]. *Cassia alata* leaf methanol extracts and *C.fistiula* leaf ethanol and hydro alcoholic extracts on *A.clavatus* and on *C. albicans* showed remarkable inhibition at 20mg/ml and also on the other fungi [28,29, 30]. *C.nigricans* leaf petroleum ether +ethyl acetate extracts in combination on *C.albicans* at 2-1MI^{-1} showed effective inhibition [31].Similarly *C.bonducella* isolated compound on *C. albicans* at 200-600 mg/ml [32].

The antioxidant activity of *P. hexapetalum* stem bark, leaf, flower, fruit and seed with aqueous, petroleum ether, ethyl acetate, hexane, acetone and methanol extracts showed equally effective action to that of the standard drug in concentration dependent manner. Flower aqueous extract at 50 µg/ml showed effective inhibition with 74.70% of free radical scavenging activity approximately equal to that of standard ascorbic acid 86.53%.[33]. The antidiarrhoeal activity of *P. hexapetalum* leaf and fruit methanol and aqueous extracts on castor oil induced diarrheal rats treated at the doses of 50 and

100 mg/kg b.wt were reduced the total number of faces as well as delayed the onset of diarrhea in a dose dependent manner in comparison to control drug *Atropine*. Enteropooling activity also very effectively reduced with fruit aqueous extracts at 100 mg/kg b.wt. as 1.4 ml and 60.45% of inhibition than 1.64 ml and 53.67% with leaf methanol extracts to that of the control drug *Atropine* with 1.68 ml and 52.54% of intestinal fluid inhibition [34]. The *in vitro* antipyretic activity of *P. hexapetalum* methanol and aqueous stem bark extracts against yeast-induced pyrexia in rats showed potential antipyretic activity. It was observed that methanol extract at a dose of 400 mg/kg body weight significantly elevated body temperature of rabbit showed maximum antipyretic activity than aqueous extract and their effects are comparable to that of standard antipyretic drug *Paracetamol* [35]

Tamarindus indica Leaf AgNPs with 18 mm and 14 mm ZOIs on *E. coli* and *Staphylococcus aureus* respectively. Results proved the significant antibacterial effect of synthesized AgNPs on bacterial strains of both gram positive and negative group [36]. The synthesized AgNPs of *Cassia tora* showed antibacterial activity against highest activity was observed on *Escherichia coli*.[37]. *Caesalpinia bonduc* AgNPs showed maximum antibacterial activity against *P aeruginosa* (120mm). Moderate activity was shown against *Salmonella typhi* (60mm), *Neisseria gonorrhea* (60mm), *Shigella dysenteriae* (50mm).The least activity was shown against *Vibrio cholerae* (35mm) and *Escherichia Coli* (40mm) [38].

CONCLUSIONS

The biosynthesized silver nanoparticles using ***Pterolobium hexapetalum*** fruit aqueous extract proved excellent antimicrobial activity against *Staphylococcus aureus* with 22.25 mm diameter zone of inhibition which also proved with the presence of significant phytoconstituents in support of ethno medicinal uses and other biological activities proved as antibacterial, antipyretic and antifungal. Hence the biological approach appears to be cost efficient alternate to conventional physical and chemical method of silver nanoparticles synthesis and would be suitable for developing a biological process for large scale production. Theses silver nanoparticles may be used in effluent treatment process also for reducing the microbial load

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