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Antimicrobial activity of the biosynthesized silver nanoparticles of *Gossypium hirsutum* leaves extract

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Abstract

Silver nanoparticles were successfully synthesized using silver nitrate and *Gossypium hirsutum* leaves extracts by varying the different concentration of aqueous & ethanolic extract. The *Gossypium hirsutum* leaves extract was containing phytoconstituents like carbohydrates, proteins, glycosides, flavonoids, alkaloids, tannins and phenolic compounds. Formation of Silver Nano Particles was primarily confirmed by colour change of yellow to brownish color. Silver nanoparticle with phytochemicals was confirmed by UV-Visible spectra by observing peak absorption of aqueous and ethanolic SNP at 430.0 nm and 416.0 nm respectively. Dynamic light scattering of the prepared formulations revealed all the formulation were within nano range, The particle size of SNP were in between 282.1nm to 205.7nm& zeta potential -38.66mV to -4.80mv for aqueous extract & particle size of SNP for ethanolic extract were in between 201.7nm to 156.1nm & zeta potential -29.95mV to -25.36mv.SEM showed the bio synthesized SNP were found to be spherical with rough surface & agglomerated. Antimicrobial activity of biosynthesized SNP was evaluated by means of inhibition zone analysis through well diffusion method, where SNP biosynthesized from aqueous and ethanolic extracts of *G.hirsutum* showed good antimicrobial activity against studied microorganisms.

Keywords: *Gossypium hirsutum*; Silver nanoparticles; Antimicrobial activity

1. Introduction

Infection is the invasion of an organism's body tissues by disease causing agents, their multiplication, the reaction of host tissues to the infectious agents and the toxins they produce. Infectious diseases are disorders caused by organisms such as bacteria, viruses, fungi or parasites. Many organisms live in and on our bodies. They are normally harmless or even helpful, but under certain conditions, some organisms may cause disease. To treat these infections antimicrobial agents are used but in recent years irrational use of antimicrobials has leads to microbial resistance, resistant microbes are more difficult to treat, requiring alternative medications or higher doses of antimicrobials. Antimicrobial resistance is a global problem that impacts all countries and all people, regardless of their wealth or status. The rise and spread of antimicrobial resistance is creating a new generation of superbugs that cannot be treated with existing medicines activity [1].

Microorganisms have evolved sophisticated mechanisms of drug resistance to avoid killing by antimicrobial molecules, microorganisms seem to have evolved a preference for some mechanisms of resistance over others. E.g. the predominant mechanism of resistance to β-lactams in Gram-negative bacteria is the production of β-lactamases. The most serious concern with antibiotic resistance is that some bacteria have become resistant to almost all the easily available antibiotics. These bacteria are able to cause serious disease and this is a major public health problem. E.g.

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Methicillin – resistant to *Staphylococcus aureus*, Vancomycin – resistsnt to *Enterococcus.* Resistance to multiple drugs was first detected among enteric bacteria namely, *E coli, Shigella* and *Salmonella* [2].

Since medicinal plants are nontoxic and easily affordable, they play a vital role not only for pharmacological research and drug development, but also when plant constituents are used directly as therapeutic agents and as starting materials for the synthesis of drugs. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids. The combination of essential oils of the medicinal plants and the standard antibiotics has significant potential for the development of new antimicrobial treatment and reduction of drug resistance, which will permit to find the treatment of several diseases caused by microorganisms. This synergy could lead to new options for the treatment of infectious diseases and emerging drug resistance activity [3].

Nano word is originated from Latin word, which means dwarf. Ideal size range offered by nanotechnology refers to one thousand millionth of a particular unit thus nanometer is one thousand millionth of a meter (i.e. 1 nm = 10^{-9} m). The branch nanotechnology is the science that particularly deals with the processes that occur at molecular level and of nano length scale size. Nanotechnology is now become an allied science which is most commonly used in other fields of science like electronic, physics and engineering since many decades. Recent exploration of nanotechnology in biomedical and pharmaceutical science results in successful improvement of conventional means of drug delivery system. SNP have been reported to exhibit number of pharmacological activities, including antibacterial activity4, antifungal activity [5], anticancer activity [6], antiviral activity [7], antiplasmodium activity [8]and mosquito larvicidal activity [9].

Since most of these chemically synthesized nanoparticles are utilized for human benefits, they pose severe hazard to human health and to the environment. Recently, green synthesis of nanoparticles using extracts of plants and microbes have gained importance because it could solve the problem of toxicity imposed by chemical methods.Green synthesis of SNP using plant extracts have been studied previously using several plants which includes *Cocos nucifera* [10], *Panax ginseng* [11], *Anacardium occidentale*[12], *Ficus benghalensis* [13], *Tribulus terrestris* [14].The studies have shown that SNP can be synthesized in environmental friendly and green method using plant extracts. Still, studies on green synthesis of SNP continue by employing medicinal plants extract to explore the potentials of traditional and medicinal plants to synthesize biocompatible SNP. The synthesis of nanoparticles has been carried out using different approaches, including physical and chemical methods. In physical methods, nanoparticles are prepared by evaporation-condensation using a tube furnace at atmospheric pressure. Chemical methods use water or organic solvents to prepare the SNP. Since, each traditional plant has specific medicinal properties; by employing these plants the medicinal property of the synthesized SNP may be enhanced. Previous studies elucidated the presence of several antimicrobial active compounds in the leaves of *Gossypium hirsutum (G.hirsutum)*. Hence, we hypothesize that the nanoparticles synthesized using *Gossypium hirsutum* leaves could be biocompatible and can be used for biological applications.

G.hirsutum belongs to the Malvaceae family. The *G.hirsutum is* most important species of the family. It has gained great importance due to its pharmacological properties including antimicrobial activity against most of the microorganisms.The *G.hirsutum* has phytochemicals such as alkaloids, glycosides, flavonoids, phenols, saponins and tannins. The phenolics, alkaloids and glycosides have antimicrobial activity. Alkaloids, saponins and tannins show strong antimicrobial activity with various antibiotics against various bacteria. So development of *G.hirsutum* silver nanoparticle may show the better anti-microbial activity than the extract [15].

2. Material and methods

2.1. Collection & extraction of *Gossypium hirsutum* leaves

The authenticated seeds of *G.hirsutum* was gifted by Mr. Rajashekhar S. Patil, ARS, Hebbali farm, Dharwad for the study, those seeds were grown in the farm in rainy season in the month of June first week in black soil by providing water. For better growth of plant homemade bio-fertilizers were provided to the plants (fig.1). After 100 days the matured leaves were collected washed with tap water for two times followed by 2% $KMnO_4$ solution. Then, double distilled water for two times and dried at room temperature in shade, then leaves were pulverized.

10gm pulverized leaves of *G.hirsutum* leaves was weighed and added to 100ml of double distilled water in 250ml in RBF and heated for 90 minutes at 75^0C. After heating the extract was filtered through muslin cloth, and again filtered by Whatman filter paper No.10 and was centrifuged at 5000 RPM for 10 minutes. Ethanolic extract was prepared by taking 10gm pulverized leaves of *G.hirsutum* in 100ml of ethanol in 250ml conical flask & kept for 24 hours in rotary flask shaker & extract was filtered by Whatman filter paper No.10. Both the extract was stored in amber colored bottle in refrigerator for further use [15, 16].

Figure 1 *Gossypium hirsutum* Plant

2.2. Phytochemical screening

The phytochemical screening of *G.hirsutum* extracts was carried out according to the methods described by Khandelwal et al [17].

2.3. Green synthesis of SNP

For the reduction of silver ions, various concentrations 1% (F1), 2% (F2), 3% (F3), 4%(F4) & 5% (F5) of aqueous & 1% (F6) 2% (F7), 3% (F8), 4%(F9) & to 5% (F10) ethanolic extract were mixed with 2mM aqueous silver nitrate in Erlenmeyer flask. A change from yellow to reddish brown color was observed after two hours shown in fig.2. To obtain of green synthesized SNP, the mixture was centrifuged at 10,000 RPM for 20 minute. The pellet was resuspended in double distilled water and centrifugation process was repeated thrice to get rid of any unreacted biological materials. The purified pellets were then dried in hot air oven at 60^0C to get SNP; these nanoparticles were further used for characterization & antimicrobial studies [18].

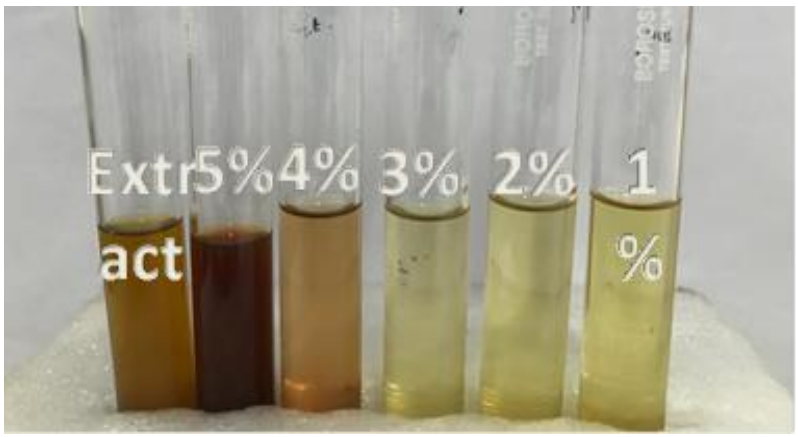

Figure 2 Change of colour from yellow to reddish brown of *G.hirsutum* leaves extract due to formation of SNP

2.4. UV spectrophotometer Analysis of SNP

In the present work supernatant containing SNP was diluted with double distilled water and the absorption was measured using a UV-Visible spectrophotometer (Shimadzu, Japan) equipped with matching cells at resolution of 1nm from 200-800nm. Double distilled water was used as blank for extract and 2mM silver nitrate was used as blank for SNP. The spectroscopic studies for SNP were carried out at room temperature [18,19].

2.5. Fourier Transform Infrared Spectroscopy (FTIR) analysis of SNP

FTIR spectra were recorded for dried biomass of *G.hirsutum* extracts before and after the bio-reduction with silver nitrate, Samples were prepared by drying the biomass at 60^0C. Solid biomass of *G.hirsutum* extract/SNP was milled with potassium bromide (KBr) to form a very fine powder was then compressed into a thin pellet which was analysed, KBr

is transparent in the IR. FTIR spectra were measured by placing the pellet in the holder of FTIR spectrophotometer (Shimadzu IR Affinity -15) [19].

2.6. Particle size and zeta potential analysis

Particle size and zeta potential of nanoparticles were determined by using dynamic light scattering (Brookhaven instrument corp). Sample preparation done by dispersing 1ml of nanoparticle solution in 10ml of dispersion medium i.e. double distilled water in glass test tube. The sample was loaded into transparent cuvette. The laser light scattering monitored at fixed angle 90 degree [19].

2.7. SEM & Energy Dispersive X-Ray Analysis (EDAX) studies

Scanning electron microscopy [SEM]study was carried out by focusing electron beam over a surface to create an image. The electrons in the beam interact with the sample, producing various signals that be used to obtain information about the surface topography and composition. Energy-dispersive X-ray spectroscopy was determined for identifying and quantifying elemental composition. The atoms on the surface were excited by the electron beam, emitting specific wavelengths of elements. An energy dispersive detector analyzed the X-ray emissions (Vega 3 Tescan) [20,21].

2.8. Antimicrobial activity

Antimicrobial activity of *G. hirsutum leaves* extracts (aqueous & ethanolic) and its synthesized SNP were evaluated by disc diffusion method, using two test Gram positive organism such as *Staphylococcus aureus* and *Streptococcus mutans,* two Gram negative bacteria *Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* and two fungus *Candida albicans* and *Aspergillus niger.* For anti-fungal disc diffusion method, sabouraud agar medium was used & for bacteria brain heart infusion agar medium was used. Inoculums of test microorganism turbidity was adjusted to 0.5 McFarland turbidity standard and cultures used were 24h fresh culture inoculated on the surface of agar plates with sterile cotton swab, agar plates were incubated at 37°C for 24h for bacteria and at 28°C for 24 h for fungal strains. After 24h wells of 6mm diameter was made with sterile cork borer & wells were loaded with the 50 µl(10mg/ml) of SNP, 2mM silver nitrate& plant extracts. After 24h of incubation efficacy of SNP, silver nitrate & *G.hirsutum leaves* extracts was determined in terms of zone of inhibition. The antibacterial activity was evaluated by measuring the diameter of the resulting zone of inhibition against the tested microorganisms in millimeters, in the present study ciprofloxacin &Fluconazole were used as standard dug [22, 23].

3. Results and discussion

3.1. Phytochemical screening, UV & FTIR spectroscopic analysis

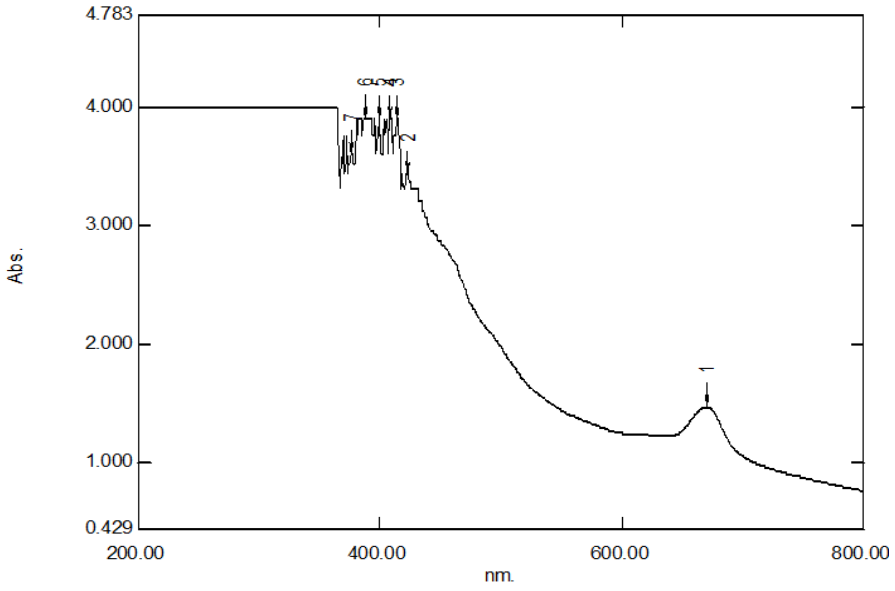

Figure 3 SNP of *G. hirsutum* aqueous extract

Phytochemical screening of *G.hirsutum* aqueous extract found to contain carbohydrates, proteins, glycosides, flavonoids, alkaloids, tannins & phenolic compounds whereas ethanolic extract found to be having steroids, glycosides, tannins & phenols.

Metal nanoparticles have free electrons, which yield a Surface Plasmon Resonance absorption (SPR) band, due to the mutual vibration of electrons of metal nanoparticles in resonance with light wave.The appearance of the peaks shows the characteristics of Surface Plasmon Resonance of SNP. The UV-Visible spectrum has the important role of silver nitrate and the presence of ingredients in the leaves for the formation of SNP. The increase in the concentration of the leaf extract will also increase the absorbance intensity. Fig.3 shows the UV-Visible absorption spectra of the SNP formed from aqueous extract of *G.hirsutum* leaves and fig.4 show sabsorbance spectra of SNP formed from ethanolic extract after 2 hours. The peak of the above spectra was due to SPR property of SNP. The synthesized aqueous SNP showed strong absorption band at 430nm for aqueous SNP and ethanolic SNP showed 416nm which is typical absorption band of SNP which was due to their surface Plasmon thus synthesized SNP may be polydispersed [24].

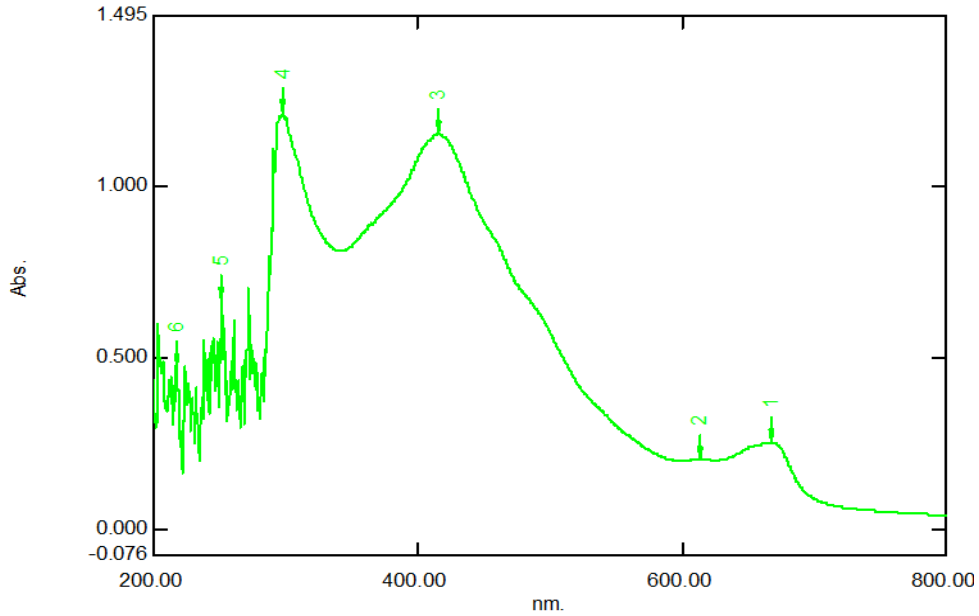

Figure 4 SNP of *G.hirsutum* ethanolic extract

FTIR analysis was used for the characterization of the extract and the resulting nanoparticles. FTIR absorption spectra of aqueous extract before and after reduction of silver ions are shown in fig 5. a. and 5.b. Absorbance bands were observed in the region of 2920.62 cm^{-1} may be due to -C-H- stretching vibration. Weak intensity of 2855.10 cm^{-1} can be assessed as absorption band of C-H stretching. 1383.81 cm^{-1} and 1633.77 cm^{-1}absorption may be due to silver. Weak intensity of 1741.59 may be due to C=O stretching. Strong intensity band of 3281.36 cm^{-1} may be due to amine group. 873.25 $cm^{-1}$and 825.00 cm^{-1} may be aromatic band.

FTIR absorption spectra of ethanolic extract before and after reduction of silver ions are shown in fig 5. c. and 5.d. Absorbance bands were observed in the region of 2919.04 $cm^{-1}$and 2853.25 cm^{-1} may be due to -C-H- stretching vibration. Weak intensity of 2555.43 cm^{-1} can be assessed as absorption band of -O-H stretching. N-H stretching at 2281.91 may observed due to amine group 1379.94 cm^{-1}absorption may be due to silver. Strong intensity of 1427.73 cm^{-1} may be due to N=O stretching. 1278.25 cm^{-1}may be –C-O- stretching. –C-O-C stretching of 1116.47 cm^{-1} and 1031.86 cm^{-1}. Strong intensity band of 3371.06 cm^{-1} may be due to amine group. Change in the position and intensity distribution of IR bands in the spectra reveals phytoconstituents for bio reduction and stabilization of SNP [25].

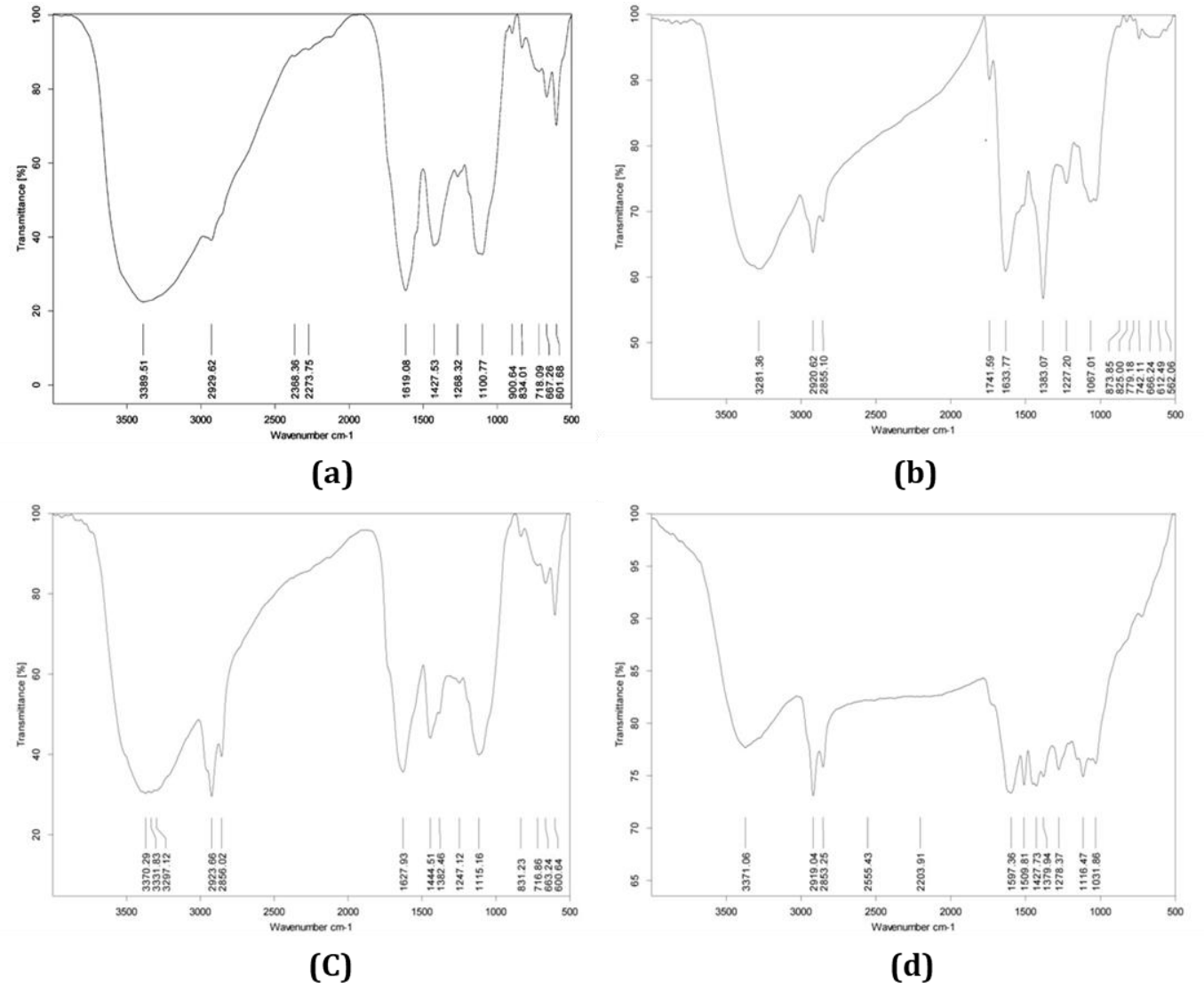

Figure 5 Aqueous extract of *G.hirsutum*b.Silver nanpoparticles after bioreduction of Aqueous extract of *Gossypium hirsutum* leavesc.ethanolic extract of *G.hirsutum* d.Silver nanpoparticles after bioreduction of ethanolic extract of *Gossypium hirsutum* leaves

3.2. Dynamic light scattering and zeta potential

The particle size of SNP was in between 282.1nm to 205.7nm & zeta potential-38.66mV to -4.80mv for aqueous extract & particle size of SNP for ethanolic extract were in between 201.7nm to 156.1nm & zeta potential -29.95mV to -25.36mv. It was observed that as the concentration of extract was increased particle size of nanoparticles was decreased & their surface charge was increased. The high negative zeta potential of the SNP confirms the repulsion among the particles and proves that nanoparticles were stable. As 5% formulation was having least average particle diameter so it was further chosen for evaluation.[18]

3.3. SEM and EDAX of Silver Nanoparticles

Primary electron can produce several secondary electrons, thus secondary electrons are abundant and the most used imaging signal in SEM. The production of backscattered electrons varies directly with the atomic number of the chemical elements present in the specimen the higher the atomic number, the brighter that region will appear [26]. SEM is a universal tool for morphological detection of SNP. The presence of SNP capped with phytoconstituents was confirmed by EDAX characterization. Fig 6.a and Fig 6.b showed the synthesized SNP were found to be spherical with rough surface & agglomeration was observed which was due to high surface charge present on nanoparticles. EDAX spectra of the synthesized nanoparticles showed an intense signal in Fig 6.c and Fig 6.d at 3.0 KeV for 5% formulation of SNP. This confirmed the presence of elemental silver which was fabricated in nanoparticles.

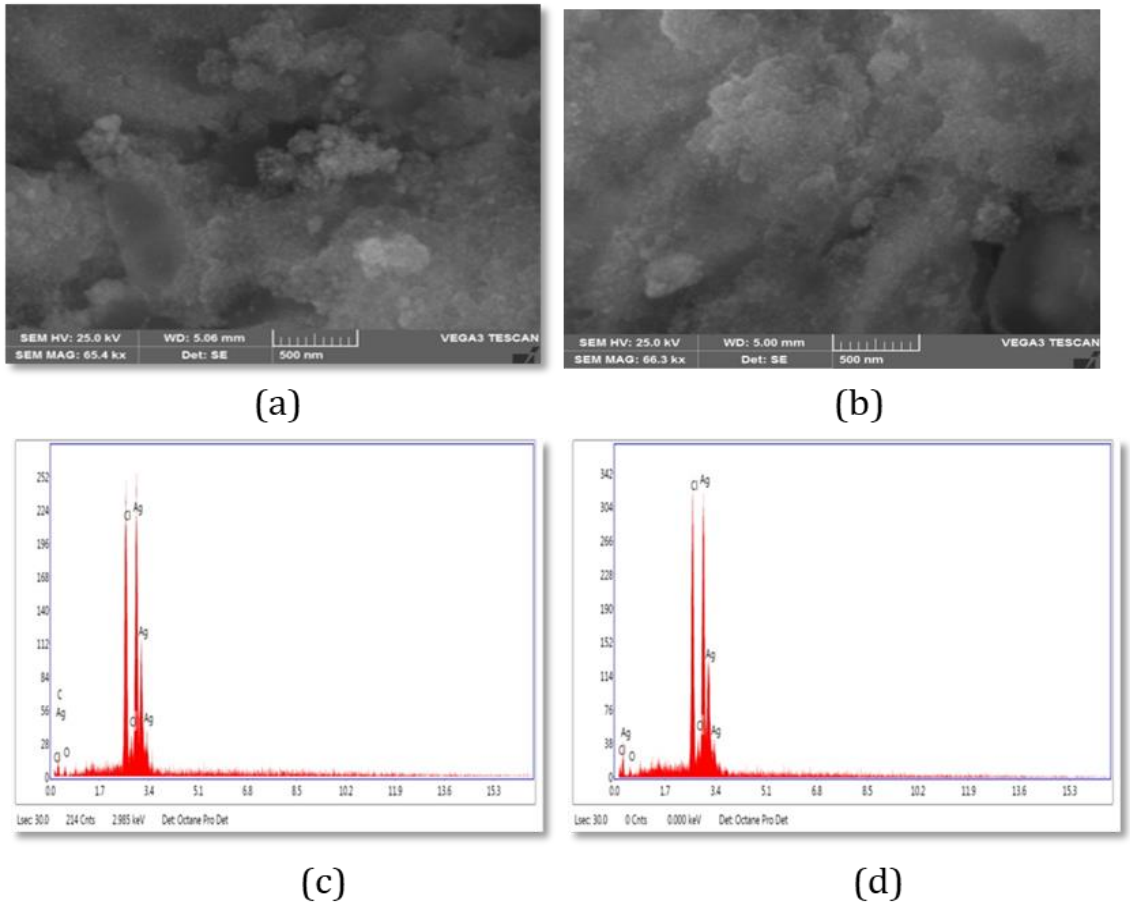

Figure 6 Extract of *G.hirsutum: a.* SEM of SNP of Aqueous extract of *G.hirsutum* b. SEM of SNP of ethanolic extract of *G.hirsutum* leaves c. EDAX of SNP of Aqueous extract of *G.hirsutum* d.EDAX of SNP of ethanolic extract of *G.hirsutum* leaves

3.4. Antimicrobial activity of *G.hirsutum* extracts Vs SNP

Table 1 Values of zones of inhibition obtained by disc diffusion method

Sr. No	Organisms	Zone of inhibition (mm)						
		Ethanolic Nanoparticles	Ethanolic Extract	Aqueous Nanoparticles	Aqueous extract	Silver nitrate	Ciprofloxacin	Fluconazole
1	*S.aureus*	16.1±0.1	Ni	15.01±0.3	Ni	8.01±0.3	45.02±0.02	-
2	*S.mutants*	15.3±0.15	Ni	14.08±0.12	Ni	10.2±0.12	30.06±0.8	-
3	*K.pneumonia*	23.1±0.12	Ni	23.2±0.16	Ni	13.0±0.8	30.01±0.07	-
4	*P.aeruginosa*	23.1±0.09	Ni	21.06±0.12	Ni	18.08±0.6	45.07±0.3	-
5	*C.albicans*	13.2±0.07	Ni	18.02±0.8	Ni	13.1±0.3	-	28.12±0.8
6	*A.niger*	18.02±0.12	Ni	15.01±0.9	Ni	12.04±0.4	-	20.09±1.1

Allthe results are mean ±SD (n=3), Ni=No inhibition

The inhibitory activity of silver and SNP had been generally perceived and applied as a valuable helpful specialist for forestalling infection. The inhibitory activity of silver on bacterial cells is due to interaction of silver with thiol bunches present in key respiratory enzymes present in the microorganisms [27]. Where it was observed that SNP should a enhanced microbial inhibition [28].

In the present investigation we have biosynthesized SNP from *G.hirsutum* aqueous & alcoholic extracts because the extracts showed good antimicrobial activity [16]. In our investigation we observed the SNP synthesized using*G. hirsutum*extract showed a pronounced zone of inhibition, this is proved by the values of diameter of zone of inhibition obtained in table.1 & fig.7 during assessment of antibacterial activity against plant extracts, silver nitrate & SNP. There was not any zone of inhibition for plant extracts at 50µl (10mg/ml) concentration, but there was significant zone of inhibition for SNP synthesized from the plant extract as compared to 2mM silver nitrate. This indicates nanoparticles where having higher zone of inhibition than silver ions, because bioreduced SNP has increased surface area hence more reactive than extract & silver nitrate.

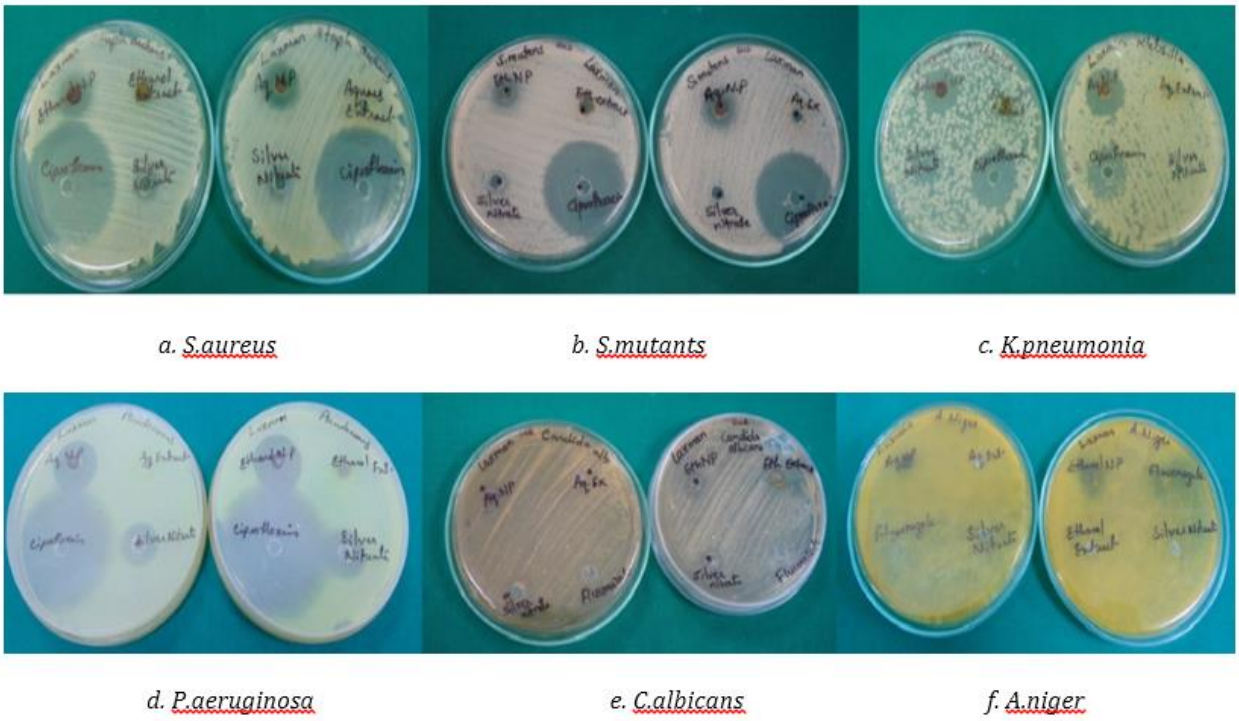

Figure 7 Plates showing diameters of zones of inhibition for Plant extracts (left aqueous extract & right ethanolic extract), Silver nitrate & SNP prepared from *G.hirsutum*

4. Conclusion

In the present study we have made an attempt to reduce the toxicity caused by chemicals used to reduce silver to from SNP by using the active constituents present in the plant extracts of *G. hisutum.* Nanoparticles biosynthesized by green synthesis in the present work were polydispersed & were characterized by different characterization techniques like DLS, EDAX, SEM, UV and FTIR, we have characterized the shape, size, crystallinity and time required to synthesize nanoparticles. Our study demonstrated the biosynthesized SNP were having good antimicrobial activity compared to the extracts of the plant.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All authors listed in this article declare that there is no conflict of interests of any kind.

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