The Pharma Innovation Journal 2019; 8(12): 376-382

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(12): 376-382

www.thepharmajournal.com
Received: 01-10-2019
Accepted: 05-11-2019

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Antimicrobial activity of biochemically synthesized silver nanoparticles (AgNPs) using *Aloe vera* gel extract

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Abstract
Biosynthesis of nanoparticles by using plant extracts is presently under development. The study has been focused on the biosynthesis of silver nanoparticles (AgNPs) using aqueous extract of *Aloe vera* gel as well as to determine their antimicrobial activity. UV-Vis spectrophotometric analysis showed surface plasmonic resonance (SPR) band at 440 nm, which is specific for AgNPs. The Transmission Electron Microscopy (TEM) revealed that the synthesized AgNPs were spherical in shape with an average particle size of 66.6 nm. Fourier Transform Infrared Spectroscopic (FTIR) analysis of the aqueous extract before and after the synthesis of AgNPs revealed the presence of different functional groups related to phenolic and polyphenolic compounds such as tannins and flavonoids, and other metabolites like proteins, which may be responsible for the synthesis and stabilization of AgNPs. The synthesized silver nanoparticles showed an excellent antibacterial and antifungal activity against the tested organisms as compared to gel extract and silver nitrate, which can gain attention of the pharmaceutical industry for preparation of antimicrobial agents of natural origin. Present study also supports the advantages of green method for the nanoparticles synthesis.

Keywords: Silver nanoparticle synthesis, Bio reduction, Aloe vera gel, Characterization, Antimicrobial Activity

1. Introduction
The increasing failures of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbes call on new site for researchers to work on prominent antimicrobial active metabolites to overcome the problem of clinically significant microorganisms including MDR microorganisms. With this respect silver and silver based compounds has been long known for its toxicity against microorganisms including bacteria and fungi. Antimicrobial property of silver is due to the fact that resistance cannot be build by the microbes against it as they are doing against conventional and narrow-target antibiotics, because it attacks a broad range of targets in the organisms, which means that microbes would have to develop a congregation of mutations simultaneously to protect themselves (Pal *et al*., 2007) [1]. To induce antimicrobial effect silver is mostly used in its nitrate form, but when nanoparticles of silver are made there is a huge increment in surface to volume ratio which makes it an excellent antimicrobial agent. Various literatures depict many ways to synthesize silver nanoparticles which include physical, chemical, and biological methods. The physical and chemical methods used for the synthesis of nanoparticles are not only energy consuming but also non eco-friendly due to the use of toxic solvents and stringent techniques. Thus efforts has been made for the development of eco-friendly and cost effective technique for synthesizing nanoparticles. So, use of plant extracts is the most adopted green and rapid method for nanoparticle synthesis because they are widely distributed, easy and safe to handle and contain several metabolites required for reduction and stabilization of nanoparticles.
Aloe vera is a unique plant which is a rich source of many chemical compounds and plays a significant role in the international market. From centuries the *Aloe vera* plant has been known and used for its health, beauty, medicinal and skin care properties. *Aloe vera* (Figure 1) is perennial succulent belonging to the Lily (Liliaceae family) (Skousen, 1979) [2] and is granted with vast array of healing benefits. About 75 nutrients and 200 biologically active compounds including sugar, anthraquinones, saponins, vitamins, enzymes, minerals, lignin, salicylic acid and amino acids has been now reported as constituents of *Aloe vera*. Previous studies suggests that the organic extracts of *Aloe vera* gel possess potent *in vitro* antibacterial activity (Saritha *et al*., 2010) [3].

Though the information on the physiological properties, antioxidant potential and antimicrobial activity of the *Aloe vera* gel are known but the antimicrobial properties of the AgNPs synthesized using it are not thoroughly worked. Present study has been carried out to assess the *in vitro* antimicrobial activity of silver nanoparticles synthesized using aqueous extract of *Aloe vera* gel.

Fig 1: *Aloe vera* plant has been known and used for its health, beauty, medicinal and skin care properties

2. Materials and Methods

2.1 Collection of plant sample: The *Aloe vera* plant was collected from Centre of Excellence on MAPs (Medicinal and Aromatic Plants) and NTFP (Non Timber Forest Products), Indira Gandhi Krishi Vishwavidyalaya, Raipur, India (21.2382º N, 81.7048º E) and was identified by Dr. P.K. Joshi, Principal Scientist and Team Leader, Centre of Excellence on MAPs and NTFP, Indira Gandhi Krishi Vishwavidyalaya, Raipur, India.

2.2 Synthesis of silver nanoparticles: Silver nanoparticles in the present study were synthesized by standard published procedures with slight modifications.

- The fully expanded leaves of *Aloe vera* was selected from three years old plant, washed with distilled water and was subjected to surface sterilization with 70% ethyl alcohol followed by 0.1% $HgCl_2$.
- The parenchymatous covering of the leaves was peeled and the gel was drained out. Slurry was formed with the help of grinder.
- Aqueous extract of the gel was prepared by using methodology as described by Niko [4] with slight modifications. 10 g of the gel formed was then mixed with 100 ml of distilled water and kept in water bath at 60ºC for 30 minutes.
- Using Whatman filter paper no. 1 the extract was then filtered and stored at 4 ºC for further use (Figure 2a).
- One mM solution of silver nitrate (Molychem, M.W 169.87, 99.9%, Thane, India) was formed by dissolving 17 mg $AgNO_3$ in 100 ml of distilled water (Figure 2b).
- AGAgNPs (AgNPs synthesized using *Aloe vera* gel extract) were synthesized by addition of 10 ml aqueous AGE (*Aloe vera* gel extract) to 40 ml aqueous 1 mM silver nitrate solution by constant stirring at 75^{0}C for 40 minutes at neutral pH (Figure 2c).
- The synthesized AGAgNPs were purified by centrifugation at 17000 rpm for 20 minutes. Purified AGAgNPs were used to determine *in vitro* antimicrobial activity.

A. B. C.

Fig 2: (a) Aqueous extract of *Aloe vera* gel, (b) 1mM $AgNO_3$ solution and (c) AgNPs synthesized using aqueous extract of *Aloe vera* gel and 1 mM $AgNO_3$ solution**.**

2.3 Characterization of the synthesized AgNPs: The bioreduction of silver ions by aqueous extract of *Aloe vera* gel was monitored by measuring the UV–visible spectrum of the reaction medium. UV–visible spectral analysis was done by using UV–visible spectrophotometer (Systronics, Double beam spectrophotometer, 2203) and the absorption maximum was scanned at the wavelength of 200-700 nm. The studies on size and morphology of AGAgNPs were performed by transmission electron microscopy. Transmission electron microscope (TEM) samples were prepared by placing a drop of dispersed AGAgNPs solution onto carboncoated copper grid. The micrographs were obtained on TECNAI G2 Spirit (FEI, Netherland) equipped with Gatan digital camera operated at an accelerating voltage at 80 kV. Fourier Transform Infrared Spectroscopic (FTIR) analysis of the aqueous extract before and after the synthesis of AgNPs was done to determine the presence of different functional groups responsible for the synthesis and stabilization of AgNPs.

2.4 Antimicrobial activity

The antimicrobial activity of the synthesized AGAgNPs was studied against 5 gram positive (*Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Bacillus cereus and Listeria monocytogenes*) and 5 gram negative (*Klebsiella*

pneumonia, Pseudomonas aeruginosa, Salmonella enteric, Escherichia coli, Aeromonas hydrophila) pathogenic bacterial strains by using agar disc diffusion method, where as poisoned food technique was used for pathogenic fungal strains (*Aspergillus terreus*, *Fusarium moniliforme*, *Aspergillus niger*, *Candida albicans* and *Histoplasma capsulatum*).

2.4.1 Agar disc diffusion method

- Agar disc diffusion method was used to evaluate antibacterial activity of AGE, AGAgNPs and $AgNO_3$ as described by (Bauer *et al*., 1966) [5]. The disc diffusion method (antimicrobial susceptibility testing) is also known as Kirby-Bauer method being recommended by the NCCLS.
- To evaluate antibacterial activity by agar disc diffusion method sterile petri plates were prepared with 20 ml of Muller Hinton Agar.
- The standard inoculums of bacterial suspension adjusted to 0.5 McFarland turbidity standard which is equivalent to 1 x 10^8 CFU/ml of bacteria were swabbed on top of the solidified media and allowed to dry for 10 min.
- With previously prepared AGE, AGAgNPs and $AgNO_3$ at concentrations of 0.25 mg/ml, 6 mm sterilized filter papers disks (Whatmann No. 1) were saturated.
- The impregnated discs were then placed onto the surface of the solidified agar medium. Petri plates were then incubated for 24 h at 35±1^{0}C.
- Finally the zone of inhibition formed by AGE, AGAgNPs and $AgNO_3$ was recorded after 24 h incubation at 35±1^{0}C. The effects were compared with that of standard, streptomycin (positive control) and distilled water was used as negative control.
- The experiment was carried out in triplicate and the results were expressed as mean values ± standard deviations.

2.4.2 Poisoned food technique

- The method of Grover and Moore (1962) [6] was adopted to evaluate the effect of AGE, AGAgNPs and $AgNO_3$ on the growth of fungi.
- 20 ml of sterilized and cooled (40-45^{0}C) growth media (PDA) with 10 mg of streptopenicillin (Gupta & Banerjee, 1970) [7] was poured into recentralized Petridishes.
- Previously prepared AGE, AGAgNPs and $AgNO_3$ at concentrations of 0.25 mg/ml were added into the plate.
- The assay plates were rotated carefully to ensure an even distribution of the extract into the medium.
- In control plates the medium was supplemented with sterilized distilled water to compensate the volume instead of AGE, AGAgNPs and $AgNO_3$.
- After solidification of agar medium inoculum of test organism (disc of 6 mm in diameter was cut from periphery of seven day old culture with the help of a sterile cork borer) was placed aseptically in the center of each Petri plate of treated and control sets.
- The assay plates were incubated at 26±1 ^{0}C for six days. After proper incubation diameter of fungal colony of treated and control sets was measured.
- The experiment was run in triplicate and the results were expressed as mean values ± standard deviations.

The percent mycelial inhibition was calculated by mean value of colony diameters by the following formula

$$\text{Percent mycelial inhibition} = dc - dt \times \frac{100}{dc}$$

Where

dc - average diameter of fungal colony in control sets.
dt - average diameter of fungal colony in treated sets.

2.5 Statistical analysis

The statistical analysis was carried out by using R. Mean and standard deviation of all the experiments was intended from triplicates (n=3) and represented in the table (Mean±S.D) and figures (error bar). Variations within the experimental groups was determined by ANOVA.

3. Results and Discussion

3.1 Characterization of the synthesized AGAgNPs: In present study the green synthesis of AgNPs using aqueous extract of *Aloe vera* gel has been elucidated. When the aqueous AGE was mixed with 1 mM $AgNO_3$ solution and incubated at 75 ^{0}C for 40 minutes at neutral pH, color changed to yellowish brown (Fig. 2c), which indicates the formation of AgNPs, this is preliminary identification of AgNPs formation. Color change in metal nanoparticles is due to the excitation of surface plasmon vibrations (Ahmad *et al*., 2003) [8]. Reaction medium containing AgNPs was subjected to UV-Vis spectral analysis where it showed sharp absorbance at 440 nm which is specific for AgNPs (Figure 3). Two control reactions (Figure 2a and 2b) were kept i.e. aqueous solution of $AgNO_3$ which was without any colour and aqueous AGE that exhibit pale yellow colour. Reaction mixture containing silver nitrate solution and aqueous AGE showed yellowish brown colouration, which indicates the formation of AgNPs. Parallel control experiments didn't show any absorption at 440 nm. TEM analysis provided details about the morphology and size of the synthesized AGAgNPs. The AGAgNPs formed was found to have an average size of 66.6 nm and spherical in shape and capped by plant constituents that prevented their aggregation. Silver nanoparticles of similar size were prepared by flower extract of *Rhododendron dauricum* (Mittal *et al*., 2012) [9]. Natural capping offers supplementary advantage of the stability in the synthesis through green chemistry route as shown in Figure 4. This stability is attributed to the phytoconstituents present in the extract, these results are in consensus with the reports by (Ahmad *et al*., 2010) [10]. The FTIR absorption spectra of the *Aloe vera* gel aqueous extract before and after the formation of AgNPs is shown in Figure 5 and 6 respectively. Absorbance bands in Figure 5 are observed in the region of 450-4000 cm^{-1} are 3366, 2933, 2123, 1633, 1423, 1372, 1251 and 1026 cm-1. These absorbance bands of the FTIR spectrum are identified to be allied with the stretching vibrations for O-H (hydrogen bonded alcohols and phenols), C-H (Alkanes), C≡C (Alkynes), C=C (Alkenes), C-H (Alkanes), C-H (Alkanes), C-N (Amines, Amides) and C-F (Aliphatic fluoro compounds) respectively which proves the presence of phenolic and polyphenolic compounds such as flavonoids and tannins, and other metabolites like proteins (Field *et al*., 2012) [11] and disappearance of these band after bioreduction (Figure 6) gives confirmation for the participation of these phytoconstituents in the formation and stabilization of AgNPs.

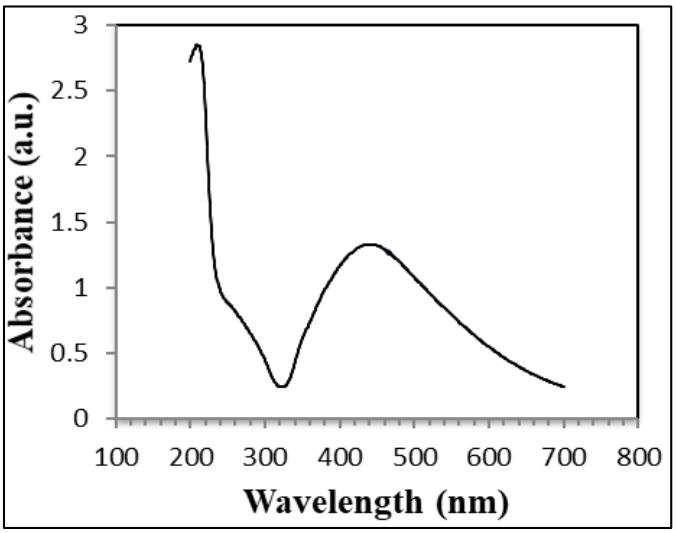

Fig 3: UV-Vis Spectrum of AGAgNPs

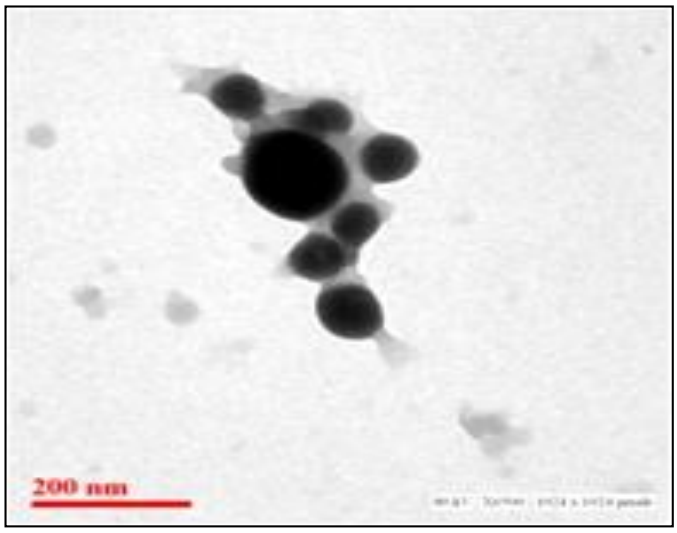

Fig 4: TEM image for AGAgNPs

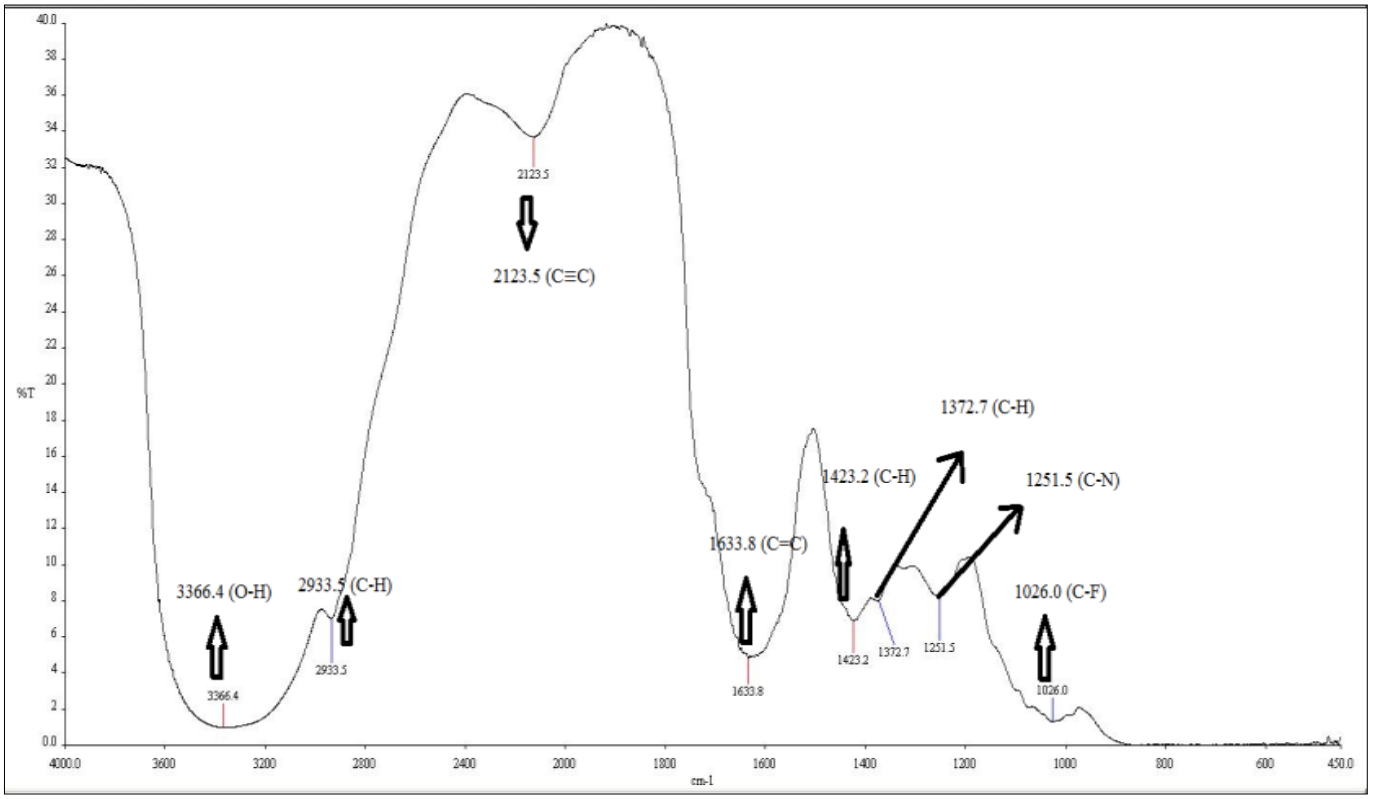

Fig 5: FTIR Spectrum of AGE

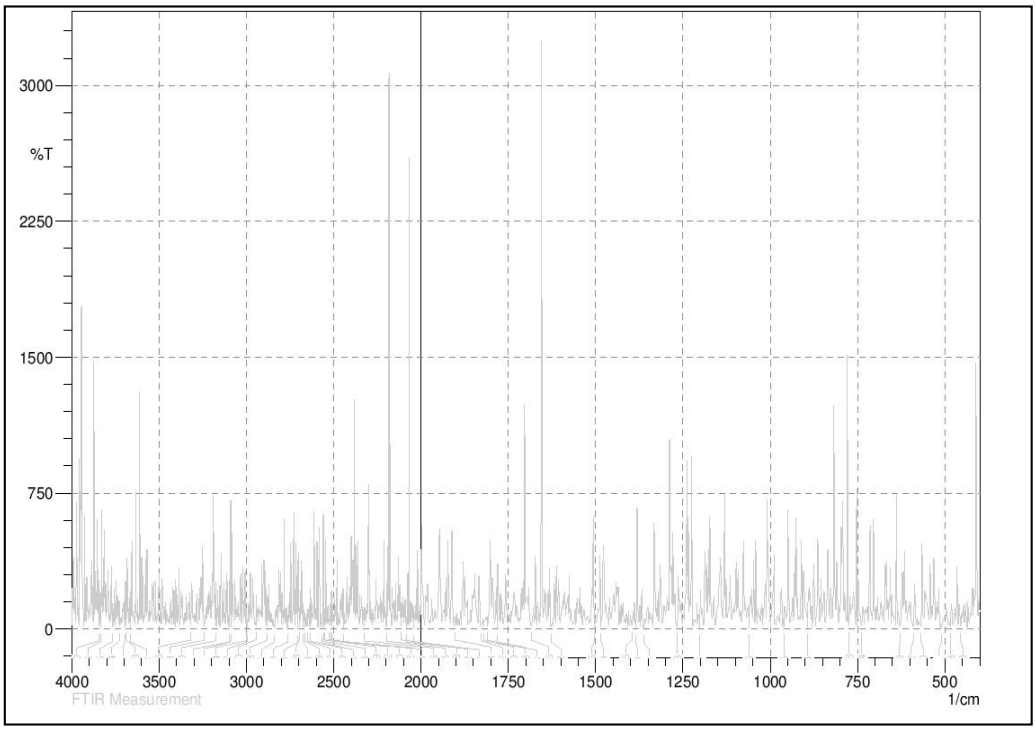

Fig 6: FTIR Spectrum of AGAgNPs

3.2 Antimicrobial activity

Broad spectrum antimicrobial activities of *Aloe vera* gel extracts has been reported (Saritha *et al.*, 2010) [3] and silver when used in its nitrate form ($AgNO_3$) is known to induce antimicrobial effect, but when silver nanoparticles are used, there is a huge increase in the surface area available for the microbe to be exposed to. So, in present study attempt has been made to synthesize AgNPs using aqueous extract of *Aloe vera* gel and aqueous solution of $AgNO_3$. Comparative antimicrobial activity of AGE, $AgNO_3$ and AGAgNPs was studied against 5 gram positive (*Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Bacillus cereus and Listeria monocytogens*) and 5 gram negative (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica, Escherichia coli, Aeromonas hydrophila*) pathogenic bacterial strains by using agar disc diffusion method, where as poisoned food technique was used for pathogenic fungal strains (*Aspergillus terreus*, *Fusarium moniliforme*, *Aspergillus niger*, *Candida albicans* and *Histoplasma capsulatum*). AGE, $AgNO_3$ and AGAgNPs were used at concentration of 0.25 mg/ml against all the microorganisms. The antibiotic streptomycin was used as a standard against bacteria in this assay. The zone of inhibition and% mycelial growth inhibition produced by AGAgNPs in plates containing bacterial lawns and fungal culture respectively was compared with plates treated with AGE and $AgNO_3$ alone. As it could be observed in table 1 and 2 and figure 7 and 8**,** the AGAgNPs showed significant antimicrobial activity against both gram-positive and gram-negative bacterial strains and pathogenic fungi as compared to AGE and $AgNO_3$. Among gram-positive bacteria *Staphylococcus aureus* and among gram-negative bacteria *Salmonella enterica* showed highest zone of inhibition i.e. 22.33±0.57 mm and 18 mm respectively ($F=886.55$, $p<0.05$) for AGAgNPs, where as in case of pathogenic fungi *Fusarium moniliforme* showed highest% of mycelia growth inhibition i.e 77.08±0.72 ($F=2927.72$, $p<0.0001$). A study carried out by (Reddy *et al*., 2014) [12] reported the enhanced antibacterial activity of green synthesized nanoparticles using *Piper longum* compared to the extract support our results.

The key to their significant microbicidal activity is due to the capability of AgNPs to release Ag+ more explicitly. The contact of AgNPs with microorganisms increases due to their high specific surface-to-volume ratio, thus promoting the dissolution of Ag^+, thereby improving microbicidal efficacy.

Table 1: Antibacterial activity of AGE, $AgNO_3$ and AGAgNPs by Agar Disc Diffusion method. Positive control = Streptomycin and Negative control = D.W. Values are expressed as Mean ± SD (n=1× 3).

S. No	Microorganism	Zone of Inhibition (mm)				
		AGE	$AgNO_3$	AGAgNPs	Control	
					Positive	Negative
1.	*Staphylococcus aureus*	15	18	**22.33**±0.57	26.33±0.57	0
2.	*Bacillus subtilis*	13.6±0.57	17	22	25	0
3.	*Bacillus cereus*	13	15.66±0.57	21	24	0
4.	*Micrococcus luteus*	12	15	20	23	0
5.	*Listeria monocytogens*	10.6±0.57	14.33±0.57	18.66±0.57	22.33±0.57	0
6.	*Salmonella enterica*	10	14	**18**	21.33±0.57	0
7.	*Aeromonas hydrophila*	10	13	16.66±0.57	21	0
8.	*Klebsiella pneumoniae*	9	12	15.66±0.57	19.66±0.57	0
9.	*Escherichia coli*	NS	11	15	19	0
10.	*Pseudomonas aeruginosa*	NS	10	13.33±0.57	15	0

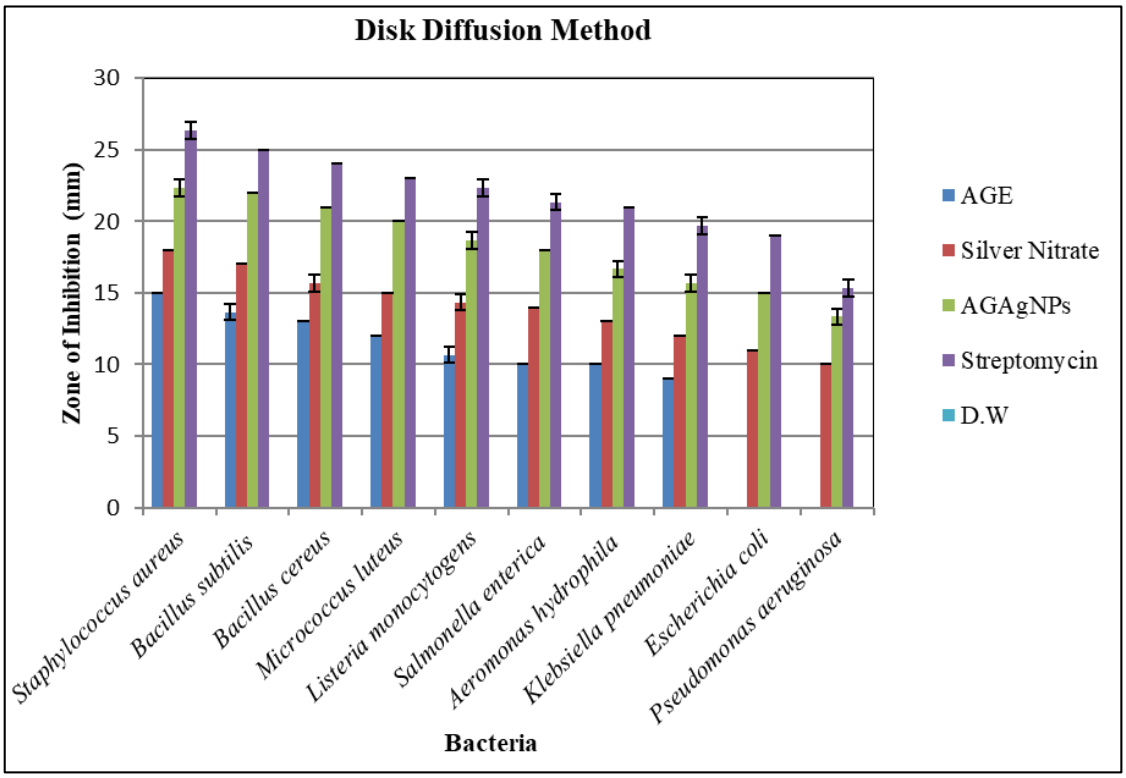

Fig 7: Antibacterial activity of AGE, $AgNO_3$ and AGAgNPs by Agar Disc Diffusion method. Each bar represent Mean±SD of three replicate observations

Table 2: Antifungal activity of AGE, $AgNO_3$ and AGAgNPs by Poisoned Food Technique. Values are expressed as Mean±SD of three individually analyzed experiments (n=1× 3).

S. No.	Microorganism	% Mycelial Inhibition		
		AGAgNPs	AGE	$AgNO_3$
1.	*Aspergillus terreus*	25.41±0.72	44.58±0.72	69.16±0.72
2.	*Fusarium moniliforme*	35.41±0.72	55.83±0.72	**77.08±0.72**
3.	*Candida albicans*	17.91±0.72	37.5	61.66±0.72
4.	*Aspergillus niger*	30	51.66±0.72	72.91±0.72
5.	*Histoplasma capsulatum*	11.6±0.72	27.5	53.75

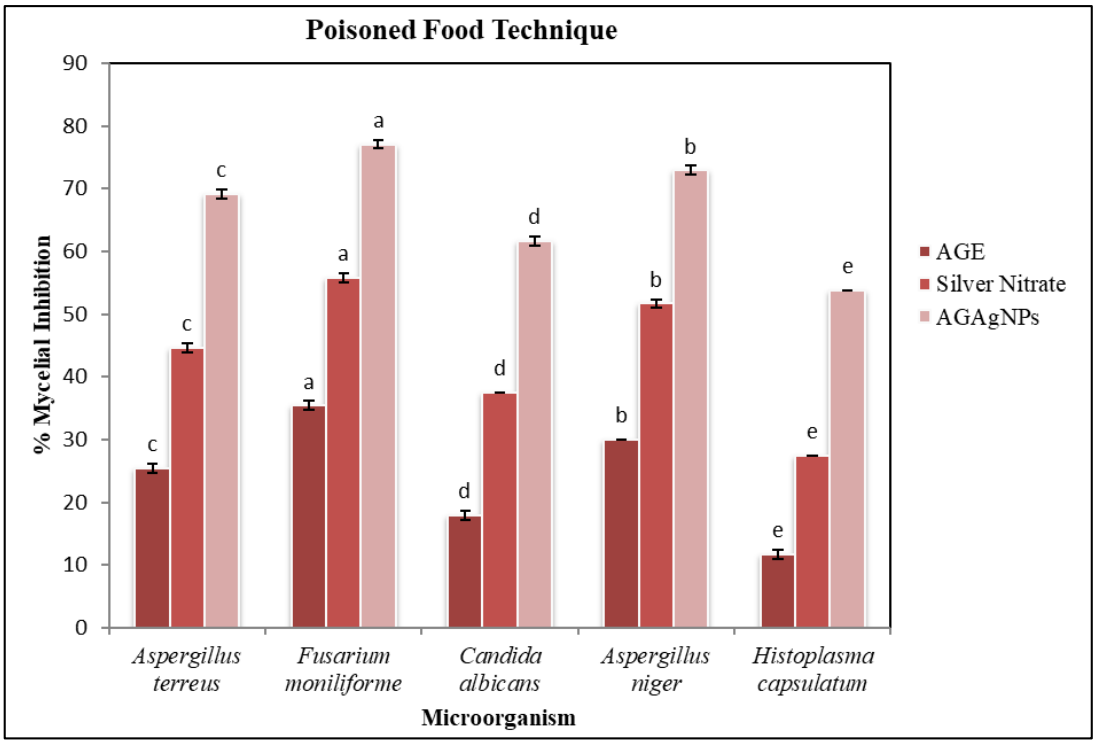

Fig 8: Antifungal activity of AGE, $AgNO_3$ and AGAgNPs by Poisoned Food Technique. Each bar represent Mean±SD of three replicate observations.

4. Conclusion

Present study reports the single step process for the biological synthesis of silver nanoparticles using aqueous extract of *Aloe vera* gel which comes up with ecofriendly, easy and proficient method for the synthesis of innocuous nanoparticles. The synthesized nanoparticles possessed the added advantage of active phytoconstituents incorporated in them. Moreover the synthesized silver nanoparticles exhibited an enhanced antimicrobial activity in all the tested organisms then the crude extract and silver nitrate, which illustrate the application of bioactive silver nanoparticles as an excellent antimicrobial agent of natural origin.

5. Conflict of interests

The authors declare that they have no conflict of interest.

6. Acknowledgement

The authors express their gratitude to the MATS school of Biological and Chemical Sciences, MATS University, for providing central laboratory facilities.

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