International Journal of Current Research and Techniques (IJCRT)
E-ISSN: 2349-3194 (Online) | P-ISSN: 2348-4446 (Print)
UGC Approved Journal No: 47722 [No: 2616]
DOI: https://doi.org/10.5281/ZENODO.18556310

Green biosynthises of nanosilver using *Gylcyrrhiza glabra* extract -screening of its antioxidant, antimicrobial and antidiabetic activity

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Manuscript Received: Dec 23, 2025; Revised: Jan 31, 2025; Published: Feb 10, 2026

Abstract: The current study aims to investigate the anti-bacterial, anti-fungal, antidiabetic and anti-oxidant properties of the silver nanoparticles synthesised by ecofriendly method of green biosynthesis by utilizing the aqueous stem extract of Glycyrrhiza glabra (Licorice Stem) as a reducing agent. The bio synthesised AgNPs are characterised in this study using visual colour change, FTIR study was used to detect the presence of functionals groups present in the AgNP, SEM analysis was used to characterise the average size of AgNPs, which were with a size range from 64.82 nm to 85.04 nm and UV-Vis spectroscopic analysis shows maximum absorbance band at 450 nm, respectively. Using the agar disc diffusion method, antibacterial and antifungal activity was evaluated against typical strains of bacteria and fungus. The antioxidant property was determined using DPPH assay and SOD assays. The antidiabetic property was tested using alpha amylase and alpha glucosidase inhibitory assays respectively and the results were compared with the results of acarbose standards. Due to its high concentration of plant-based phytoestrogens and/or natural antioxidants, the legume Glycyrrhiza glabra may be used in the long run to treat and/or prevent diseases linked to oxidative stress including diabetic nephropathy, ferroptosis and certain types of cancer and nanobiomedial applications.

Keywords: Glycyrrhiza glabra, antidiabetic property, natural antioxidants, antibacterial activity, alpha amylase, alpha glucosidase

1. Introduction:

Right from the discovery of colloidal ruby gold nanostructure till the synthesis of quantum dots numerous researches has been conducted on nanoparticles. Nanotechnology has become one of the most prominent areas of research in the field of medical science and material research. Both the chemical and physical processes used to synthesize nanoparticles were not sustainable, which led to the necessity for the alternative process, biosynthesis of nanoparticles1. The poor efficiency and presence of harmful substances in the process of chemical and physical synthesis of nanoparticles created a need for a process which is environmentally friendly, cost effective with a practical biosynthetic approach. In this approach, either biological microbes or plant extracts were used as a better alternative2 as this process doesn't involve any harmful toxicants.

Green biosynthesis of nano particles is considered to be advantageous over other biosynthesis methods such as synthesis of nanoparticles using bacterial cultures which requires the laborious task of maintenance of the viable culture usually prone to contamination. The phytochemicals present in the plant extract are often the once that reduces the metal oxides into their corresponding nanoparticles with the help of their reducing or anti-oxidant properties3. The appealing physio-chemical nature of silver nanoparticles have made them highly valuable in various fields of biology and medicine. The light absorption efficiency, and colour of the silver nanoparticles is based on their size and form. The cytotoxicity, inflammation, and genotoxicity effect of the nanoparticles too depends on the size and shape of the AgNPs synthesised4. Numerous analytical techniques, such as X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), ultraviolet visible spectroscopy (UV-vis spectroscopy), dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and others, have been used to assess the properties of synthesized nanomaterials5. The synthesis of silver nanoparticles using the aqueous extract of Glycyrrhiza glabra stem extract as a reducing agent to reduce the silver ions in the silver nitrate solutions is reported here along with its anti-oxidant, antibacterial and antidiabetic properties. The synthesised silver nanoparticles (AgNPs) were characterized using FTIR, SEM, UV absorption techniques and visual examination (colour change).

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2. Materials and Methods

Plant Material and Preparation of the Extract

Dried stem of licorice was collected, washed thoroughly with distilled water, incised into small pieces, dried and powdered. About 25 g of powdered stem sample were weighed and the solvent taken (ethanol) is poured on top till the solute is completely immersed. The container is then closed and kept for 5 days while it is periodically shaken to ensure complete extraction. The extract was filtered using Whatman filter paper and condensed using rotary evaporator in reduced pressure at 50ºC. The extract was tested for the phytochemical analysis as these phytochemicals are the essential elements that help in the reduction of silver nitrite to its nanoparticle form. This extract was used for Ag NPs synthesis and stored at 4°C for further experiments.

Synthesis of AgNPs

About 10 mg of licorice stem extract was dissolved with 1ml of dimethyl sulfoxide (DMSO) for the green biosynthesis of AgNPs. A magnetic stirrer was used to stir this with 200 ml of 1 mM aqueous solution of $AgNO_3$ for 48 hours. The color change from colorless to brown indicated the reduction of silver nitrate to silver ions. This color change is due to the surface plasmon resonance property of the spherical silver nano particles synthesised[6]. Spectrophotometric analysis was another method used to verify the generation of silver nanoparticles. The solution was thoroughly reduced and centrifuged for 30 minutes at 5000 rpm. The supernatant solution was discarded after which the particle obtained were redispersed in deionized water.

UV-Vis Spectroscopy

The biogenic synthesis of AgNPs was monitored periodically by UV-Vis spectroscopy. All spectra were collected by scanning from 350 to 500 nm ranges. The nanoparticle solution showed maximum absorbance at 450 nm.

Antioxidant Assay

DPPH ASSAY

Using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl), the free radical scavenging activity of AgNPs was evaluated. For DPPH assay[7] aliquot 3.7 ml of absolute methanol in all test tubes and 3.8ml of absolute methanol was added to the test tube marked as blank, 100 µl of BHT was added to tube marked as standard and 100 µl of respective samples were added to all other tubes marked as tests. 200 µl of DPPH reagent was added to all the test tubes including blank. Test tubes were incubated at room temperature in dark condition for 30 minutes. The absorbance of all samples was read at 517 nm. Using the following formula, the percentage of inhibition representing the free radical scavenging activity was calculated.

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

Assay of Superoxide dimutase (SOD)[8]

The assay mixture contained 1.2 ml of Sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT (Nitroblue Tetrazolium Test), 0.3 ml of the sample and1ml of water was added. The reaction was initiated by the addition of 0.1 ml of NADH (nicotinamide adenine dinucleotide hydrogen). The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 0.1 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50 % inhibition of NBT reduction on one minute.

$$\% \text{ Antioxidant activity} = \frac{(\text{Absorbance at blank}) - (\text{Absorbance at test})}{(\text{Absorbance at blank})} \times 100$$

Antidiabetic Assay

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Determination of alpha-amylase inhibitory activity

For this assay[9] the assay mixture was prepared containing 200 µl of sodium phosphate buffer, 20 µl of enzyme and 20 µl of extracts and incubated for 10 minutes at room temperature followed by the addition of 200 µl of starch in all the tubes. The reaction was terminated with the addition of 400 µl of DNS reagent and placed in boiling water bath for 5 minutes, cooled and diluted with 15 ml of distilled water and absorbance was measured at 540 nm. The control samples were prepared without any extracts. The % inhibition was calculated by following the formula.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Determination of alpha-glucosidase inhibitory activity

Alpha-glucosidase inhibitory activity of extracts was carried out according to method[10] with slight modification. Reaction mixture containing 50 µl phosphate buffer, 10 µl alpha-glucosidase and 20 µl of varying concentrations of extracts was pre-incubated at 37ºC for 15 min. Then 20 µl p-nitrophenyl-α-D-Glucopyranoside (PNPG) was added as a substrate and incubated further at 37ºC for 30 min. The reaction was stopped by adding 50 µl sodium carbonate. The yellow color produced was read at 405 nm. Each experiment was performed along with appropriate blanks. Acarbose at various concentrations (20-100 µg/ml) was included as a standard. Negative control without extracts was set up in parallel. The result is expressed as percentage inhibition.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Antifungal Assay

Using sterile cotton swabs, *Candida albicans* and *Trichoderma viride*, *Penicillium chrysogenum* were inoculated on Potato Dextrose Agar medium and the antifungal activity was studied by loading 20 µl of 1000 µg/ml concentration of the extract and nanoparticles synthesised were loaded on to the sterile disc. For comparison, Amphotericin B and distilled water were used as positive standard and negative control respectively. The diameter of the zone of inhibition surrounding the disc was measured to determine the antifungal activity.

Antibacterial Assay

The agar disc diffusion assay of the Kirby-Bauer disk diffusion technique was used to evaluate AgNPs antibacterial activity. Strains used in this study were gram positive bacteria's such as *Staphylococcus aureus, Bacillus cereus*, gram negative bacteria's such as *Escherichia Coli, Klebsiella pneumoniae, Vibrio cholerae* and *Salmonella enterica*. Using sterile cotton swabs, the above said bacterial species were inoculated on Mueller Hinton Agar medium, 20 µl of 1000 µg/ml concentration of the extract and nanoparticles synthesised were loaded on to the sterile disc. For comparison, ampicillin and distilled water were used as positive standard and negative control respectively. On to the inoculated plates the discs were placed and incubated at 37°C overnight. The diameter of the zone of inhibition surrounding the disc was measured to determine the antibacterial activity.

3. Results and Discussion

Synthesis of silver nanoparticles

The silver nanoparticles were efficiently synthesized from licorice stem extract with 1 Mm $AgNO_3$ containing solution at room temperature. About 10 mg of *Glycyrrhiza glabra* stem extract was diluted with 10 ml of dimethyl sulfoxide (DMSO) for the green biosynthesis of AgNPs. A magnetic stirrer was used to stir this with 200 ml of 1 mM $AgNO_3$ until the colour changes from an opaque to brown then to a dark brown solution. AgNPs formation was indicated by the reaction mixture's colour change (Fig.1). The control solution without the extract didn't show this colour change.

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Fig.1 Visual confirmation (A) 1mM silver nitrate with aqueous stem extract (B) 1 mM silver nitrate solution after reaction with licorice stem extract after 24 hours (C) 1 mM silver nitrate solution after reaction with licorice stem extract after 48 hours

4. Characterization of silver nanoparticles

The characterization of AgNPs synthesized by the green method was confirmed using UV-Vis spectroscopy. The results of the UV-Vis spectroscopy show a continuous increase followed by a peak at 450 nm that subsequently slowly decreases, forming a bell-shaped curve that confirms the presence of silver nanoparticles and demonstrates the effectiveness of the extract's bioactive ingredients in reducing the silver nitrite to silver ions (Fig 2). The UV Vis spectroscopy is the most practical technique for assessing the reduction of metal ions based on optical properties termed (SPR) surface plasmon resonance[11]. Similar UV–Vis absorption spectra was reported in various studies AgNPs were synthesized from *Citrus reticulata* peel extract, *Ficus benghalensis*, *C. circinalis*, *F. amplissima*, *C. benghalensis* and *L. Nodiflora* leaf extracts and *Ficus cariaca* dried fruit extract, with an absorbance peak centered between 400–450 nm.[12, 13]

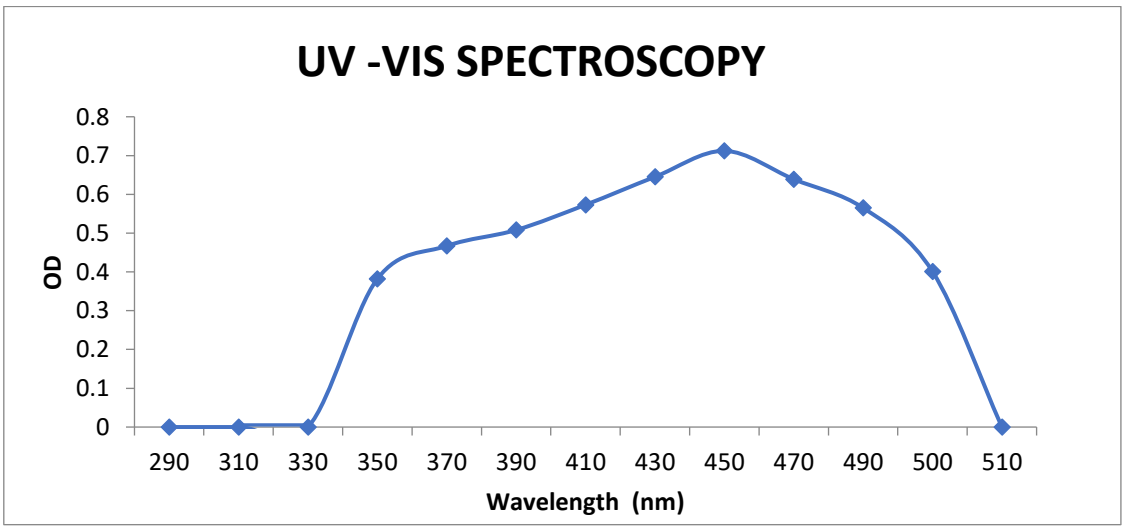

Fig.2 The synthesized AgNPs showed absorbance spectra at 450 nm in UV -VIS spectroscopy

FTIR analysis

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AgNPs synthesized with licorice stem extract were examined using FTIR, and the results were displayed in Fig.3. The peak values in the infrared radiation region of the graph indicate the functional groups. With a Perkin-Elmer spectrometer, the FTIR spectra was captured at a resolution of 4 cm^{-1} and in the range of 3329.88–1010.51 cm^{-1}. In order to analyze the nanoparticles, a thin sample disc was made and placed in a fourier transform infrared (FTIR) apparatus. A wide band at 3329.88 cm^{-1}, corresponding to O–H group stretching vibrations. Stretching of C≡ C is represented by the bands at 2,161.92 cm^{-1}. A peak has been seen in the low wavelength range at 1636.94 cm^{-1}, which is the amide-I band (C=O stretch of ester group). We reasoned from these peaks that the production of Ag NPs involves both the hydroxyl and carbonyl groups of the licorice stem extract. These peaks closely resemble those seen in other scientific examinations on green synthesis of silver nanoparticles[12 13]. Results of FTIR Spectroscopy indicated the existence of a variety of biomolecules that are essential for the stabilization of AgNPs (Fig.3).

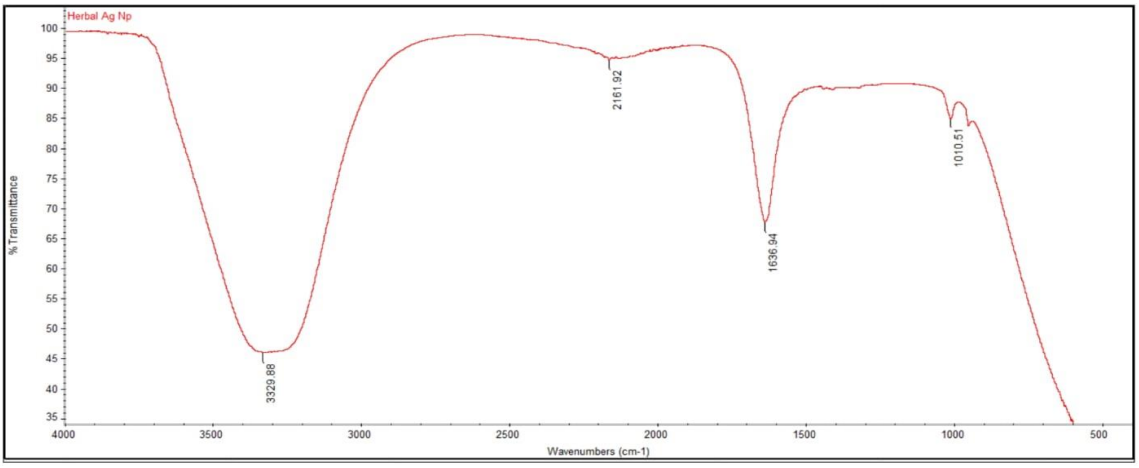

Fig.3 FTIR analysis of green biosynthesized AgNPs showing peaks representing the presence of different functional groups.

SEM analysis

Using scanning electron microscopy (Vega 3 tescan), the morphology of the silver nanoparticles was investigated, and images were generated at 15 kV and 10kV. SEM images show that the produced silver nanoparticles were uniformly distributed throughout the colloidal mixture with a size range from 64.82 nm to 85.04 nm (Fig.4).

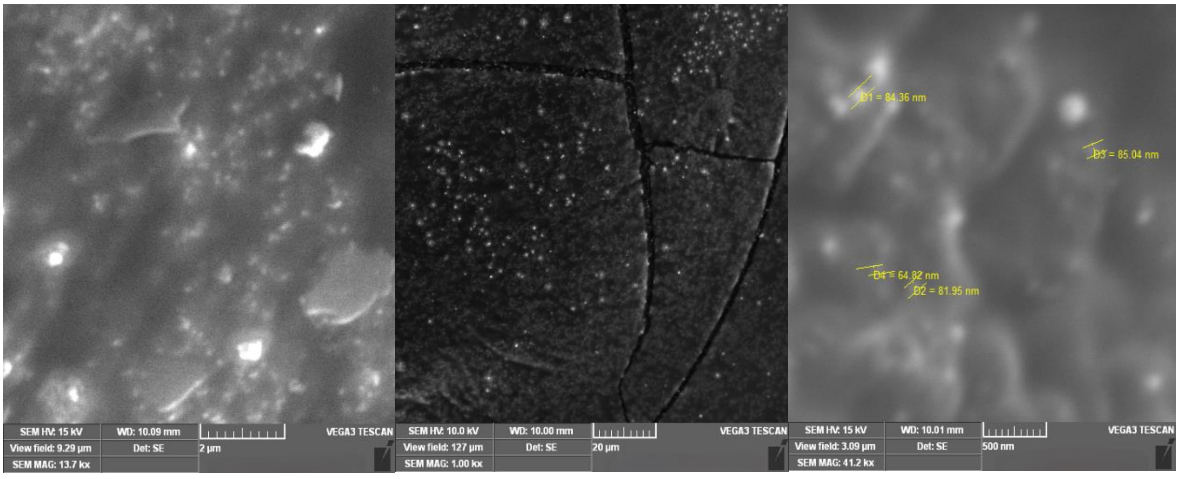

Fig.4 SEM analysis of green biosynthesized AgNPs.

Antioxidant assay

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DPPH Assay

The outcome of this assay is likely to vary based on the characteristics of the scavenging molecule in the sample. The results of the assay showed that at increasing AgNPs concentrations, there was a significant rise in the amount of DPPH free radicle scavenging activity. The obtained results indicated that the generated AgNPs possessed strong antioxidant properties and the results were compared with Butylated Hydroxytoluene (BHT) standard (Tables.1 & 2; Fig.5).

Table.1 DPPH Activity of synthesised AgNP

S.NO	Concentration (µg/ml)	O.D	DPPH %
1	200	0.320	20.39
2	400	0.254	36.81
3	600	0.184	54.22
4	800	0.116	71.14
5	1000	0.049	87.81

Control- 0.402

Table.2 DPPH Activity of BHT Standard

S.NO	Concentration (µg/ml)	O.D	DPPH %
1	200	0.100	60.31
2	400	0.086	65.87
3	600	0.072	71.42
4	800	0.053	78.96
5	1000	0.033	86.90

Control- 0.252

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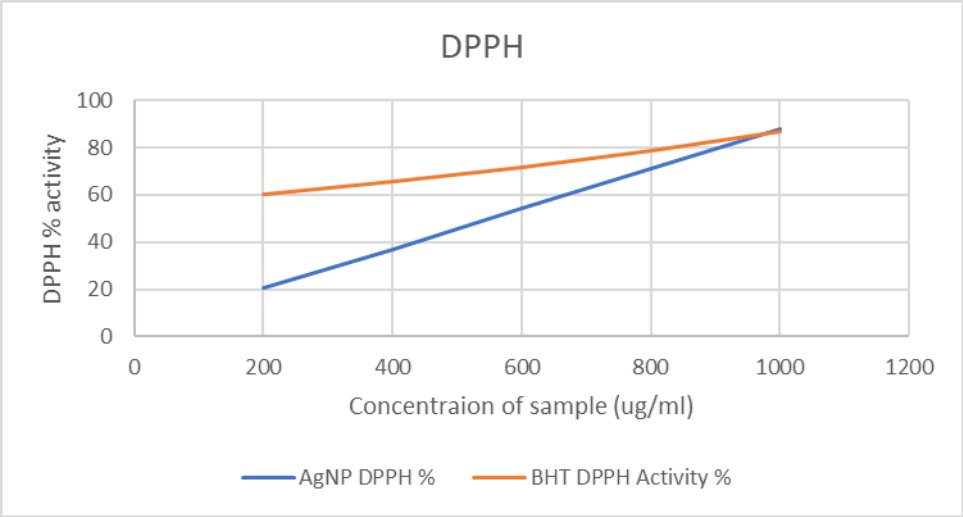

Fig.5 DPPH % Activity with increasing concentration of sample and standard

Superoxide dimutase (SOD) assay

The superoxide radicle scavenging activity of potential antioxidant property of the AgNPs synthesised using the plant extract is tested using the SOD assay in this study. The assay's findings demonstrated that when AgNPs concentrations increased, so did the antioxidant property (Table.3; Fig.6).

Table.3 SOD Activity of synthesised AgNP

S.NO	Concentration (µg/ml)	O.D	SOD %
1	200	0.059	20.27
2	400	0.047	36.48
3	600	0.035	52.70
4	800	0.021	71.62
5	1000	0.007	90.54

Control- 0.074

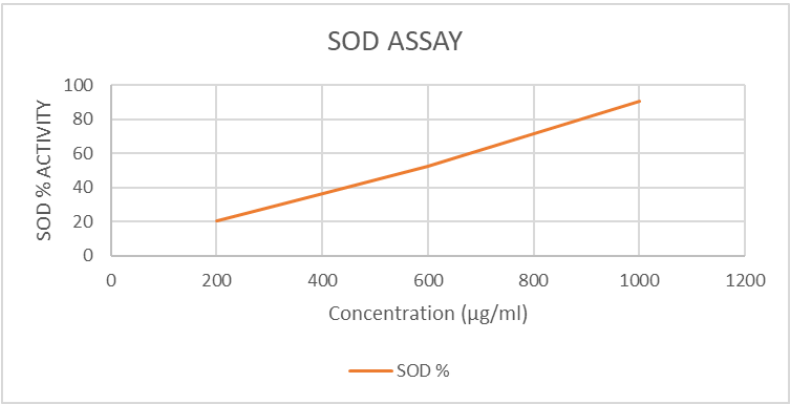

Fig.6 SOD Activity of sample on increasing concentration

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Antidiabetic assay

Inhibition of the enzymes like alpha amylase and alpha glucosidase was helpful for the treatment of diabetes and obesity as these where the enzymes that were responsible for the breakdown of oligosaccharides and disaccharides into simple sugars. Hence their inhibition leads to delaying the sugar spike in the blood stream. Phytochemicals such as saponins, terpenoids and phenolic compounds are the natural pancreatic lipase inhibitors present in plant extract [14, 15]. The current study examined the alpha-glucosidase and alpha-amylase inhibitory activity of AgNPs synthesised using liquorice stem extract, the findings are displayed in the table below. The *in vitro* assay indicates that the AgNPs exhibited varying inhibitory effects on α-amylase and α-glucosidase. The α-glucosidase inhibitory assay generally indicates that, in contrast to acarbose[16], the green synthesized AgNPs were stronger in inhibiting the activity of α-amylase(Tables.4&5; Fig.7) and α-glucosidase(Tables.6&7; Fig.8)in higher concentrations. This result aligned with the findings of Shai *et al.*,[17] who documented the α-glucosidase inhibitory effect of acarbose standard.

Table.4 Alpha –Amylase Inhibitory Activity of synthesised AgNP

CONCENTRATION OF SAMPLE (µg/ml)	O.D. Value	% INHIBITION
20	0.378	34.82
40	0.302	47.93
60	0.226	61.03
80	0.150	74.13
100	0.074	87.24

Control- 0.146

Table.5 Alpha –Amylase Inhibitory Activity of Acarbose Standard

CONCENTRATION OF SAMPLE (µg/ml)	O.D. Value	% INHIBITION
20	0.096	34.24
40	0.073	50
60	0.054	63.01
80	0.032	78.08
100	0.016	89.04

Control 0.580

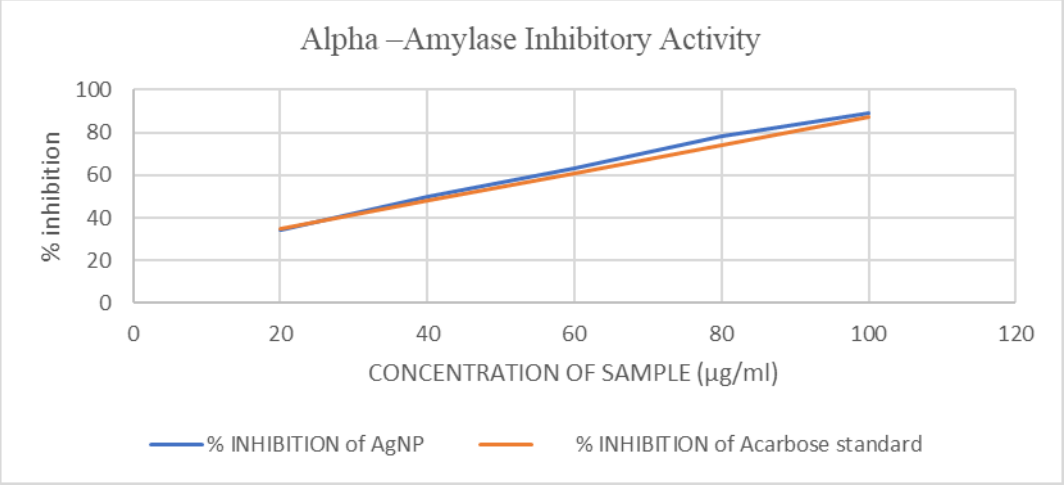

Fig.7 Alpha –Amylase Inhibitory Activity of synthesised AgNP and acarbose standard.

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Table.6 Alpha – Glucosidase Inhibitory Activity of synthesised AgNP

CONCENTRATION OF SAMPLE (µg/ml)	O.D. Value	% INHIBITION
20	0.344	8.51
40	0.260	30.85
60	0.174	53.72
80	0.091	75.79
100	0.011	97.07

Control -0.376

Table.7 Alpha – Glucosidase Inhibitory Activity of Acarbose Standard

CONCENTRATION OF SAMPLE (µg/ml)	O.D. Value	% INHIBITION
20	0.101	30.34
40	0.082	44.44
60	0.063	56.55
80	0.044	69.65
100	0.025	82.75

Control- 0.145

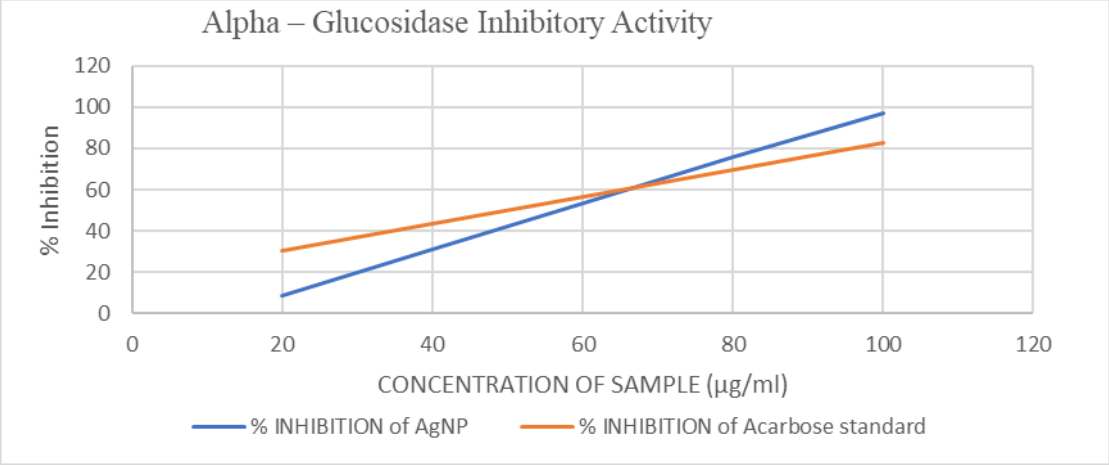

Fig.8 Alpha –Glucosidase Inhibitory Activity of synthesised AgNP and acarbose standard.

Antibacterial activity

It has been shown that Ag-NPs work well as a broad-spectrum antibiotic against both Gram-positive and Gram-negative bacteria[18]. Furthermore, the nanoparticles synthesized by green route are found to be highly effective against multi-drug resistant human pathogenic bacteria[19]. Antibacterial activity of silver nanoparticles synthesised against the earlier mentioned gram positive and negative bacteria were investigated and compared with the standard drug and the licorice stem extract (Fig 9). The zone of inhibition was observed and given in table below.

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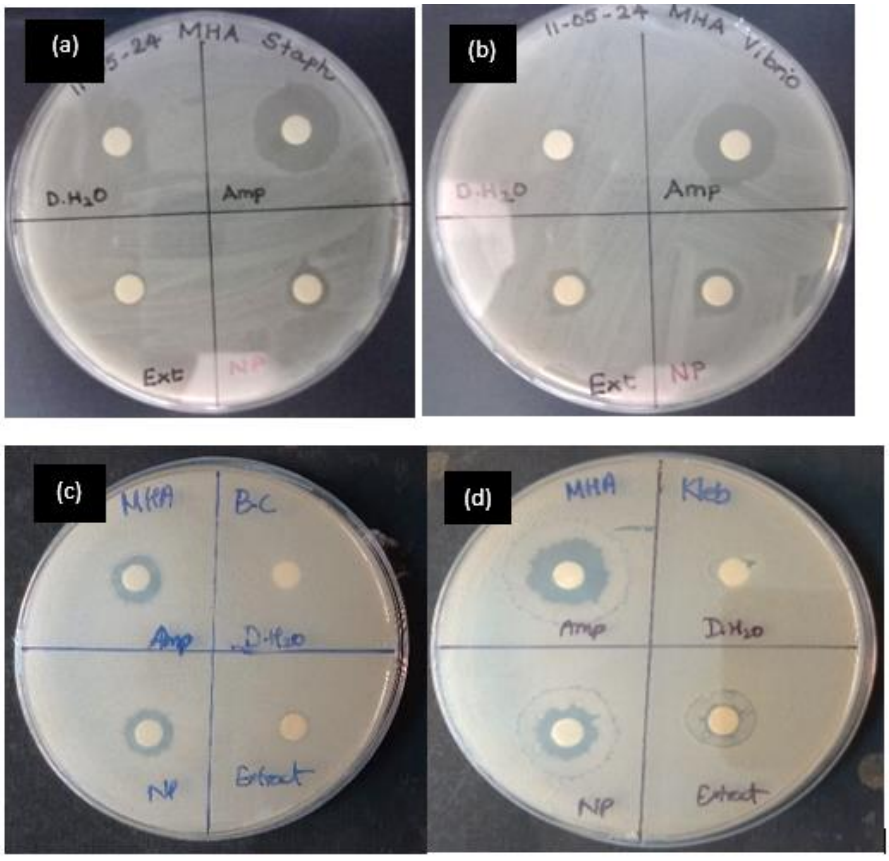
Table.8 Antibacterial Activity of control, extract and AgNP

S No	STRAIN	POSITIVE CONTROL	NEGATIVE CONTROL	LICORICE EXTRACT	SILVER NANOPARTICLES
	Culture	Ampicillin(20µl)	D.H_2O (20µl)	1000µg (20µl)	1000µg (20µl)
1	*Staphylococcus aureus*	18	-	7	9
2	*Bacillus cereus*	11	-	8	11
3	*Escherichia Coli*	18	-	9	12
4	*Klebsiella pneumoniae*	15	-	8	12
5	*Vibrio cholerae*	16	-	8	10
6	*Salmonella entrica*	18	-	8	10



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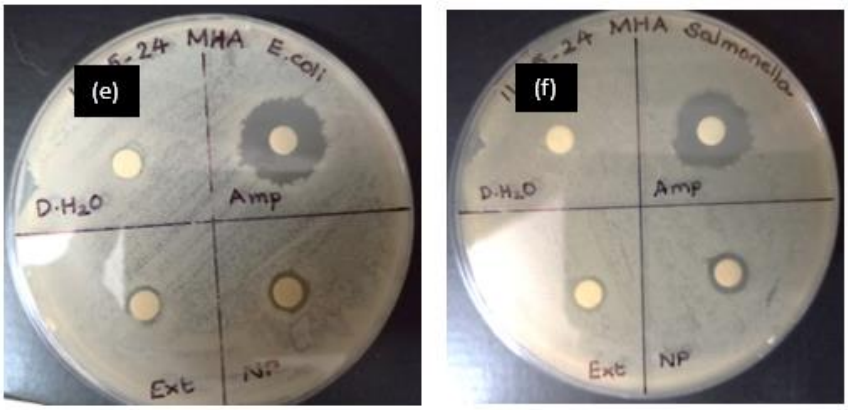

Fig.9 Antibacterial activity of biosynthesized silver nanoparticle extract and standard against (a) *Staphylococcus aureus* (b) *Vibrio cholerae* (c) *Bacillus cereus* (d) *Klebsiella pneumoniae* (e) *Escherichia Coli and* (f) *Salmonella enterica*

Antifungal activity

As per the findings of Kim *et al*.,[20] nano-Ag shown considerable antifungal properties against certain fungi by damaging their membrane integrity and interfering with their cell division. The antifungal potential of the AgNPs was compared with standard drug amphotericin b and licorice stem extract (Fig.10). The zone of inhibition was observed and given in the table below (Table.9).

Table.9 Antifungal Activity of control, extract and AgNP

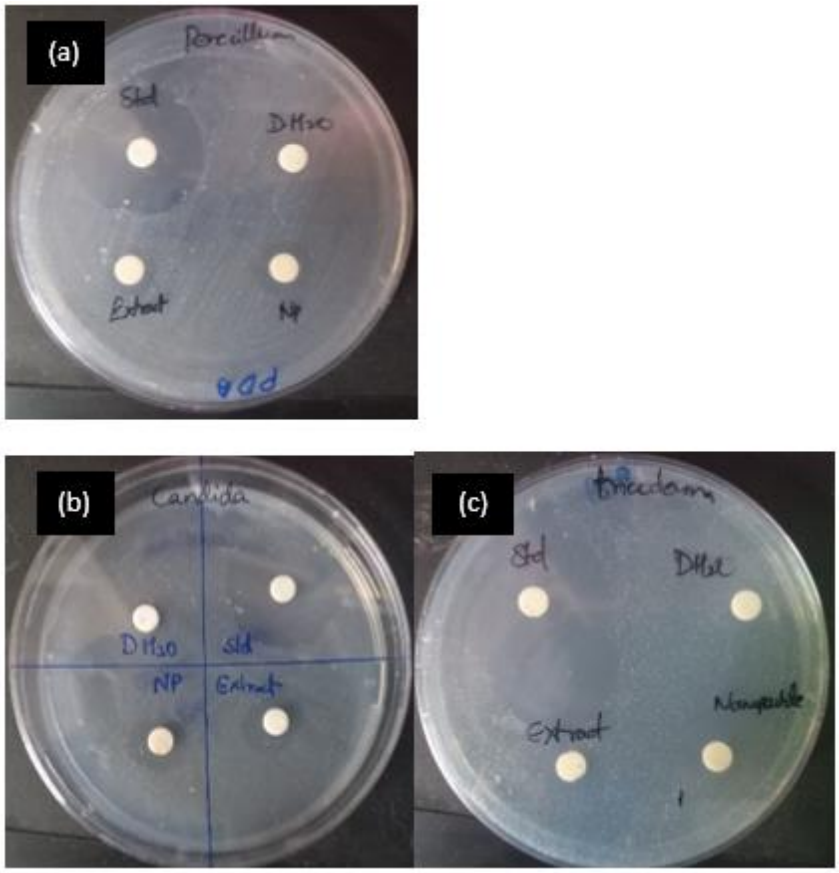

Fig.10 Antifungal activity of biosynthesized silver nanoparticle, extract and standard against (a) *Penicillium chrysogenum* (b) *Candida albicans* and (c) *Tricoderma viride*

5. Conclusion

The potential of AgNPs for their use as drug carriers in cancer therapy, as biosensors for metabolites and pollutants, as catalyst etc made it a subject of interest in the field of scientific research. It requires intensive and integrated research activity for harnessing it. The formation of AgNPs was triggered by the addition of licorice stem extract to silver nitrate solution due the catalytic properties of hydroxyl and carbonyl groups present in the extract. For the creation of nanoscale inorganic materials, this environmentally benign method of green biosynthesis of AgNPs is a competitive substitute for physical and chemical methods which releases harmful toxicants as byproducts. The synthesised AgNPs are characterised and various properties of this AgNPs produced by this environment friendly method was studied and the results were compared with the standards and given in the results. This study shows the significance of green biosynthesis of silver nano particles and its antimicrobial, antioxidant and antidiabetic activity. The biomedical and agricultural application of this AgNPs is yet to be studied.

6. Acknowledgement

The authors would like to thank the Principal of Pachaiyappa's College, Chennai, for providing the necessary facilities to conduct this work.

7. Funding Sources

This project did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

8. Conflict of Interest

Authors do not have any conflict of interests to declare

9. Data Availability Statement

This statement does not apply to this article.

10. Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

11. Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

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