Research Article

Glycirrhiza glabra as a Potential Synthesizer of Silver Nanoparticles and their Microbicidal Action

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ABSTRACT

Nanoparticles are gaining interest in biomedical applications due to its importance such as anti-bacterial, anti-fungal and anti-cancer agents. Silver nanoparticles were formed when the reaction conditions were altered with respect to concentration of silver nitrate, consumption of hydrazine hydrate, ALE content and incubation temperature. Plant extracts of *Glycirrhiza glabra* are cost effective and economically used for synthesis of nanoparticles. The colorless reaction mixture turned brown and displayed UV–visible spectra characteristic of silver nanoparticles at 240 nm. FTIR analyses suggest that the plant compounds such as flavonoids and terpenoids were responsible for the formation of silver nanoparticles. Hplc analysis indicates the presence of glabridin responsible for antimicrobial efficacy. Inhibitory effect was found to be maximum in 400 µl of synthesized Agnps against Bacillus sp. (24 mm), S. epidermis (23 mm), P. aeruginosa (22 mm).

Keywords: Green synthesis, Absorption spectrum, Bioactive constituents, Microbicidal, Inhibition.

INTRODUCTION

Nanotechnology is an emerging field of science due to their wide application in biological fields. Frequently, nanometer-size metallic particles show unique and considerably changed physical, chemical and biological properties compared to their macro scaled counterparts, due to their high surface-to-volume ratio. Thus, these nanoparticles have been the focus for significant research in recent years[1-4]. Metallic nanoparticles exhibit size and shape-dependent properties that are of interest for applications ranging from catalysts and sensing to optics, antibacterial activity[5-10]. The biosynthesis of nanoparticles has been proposed as a cost-effective environmental friendly alternative to chemical and physical methods. For example, the antibacterial activity of different metal nanoparticles such as silver colloids is closely related to their size; that is, the smaller the silver nuclei, higher the antibacterial activity.

Recently, some studies have shown that specially formulated Ag-NPs have good antibacterial activity[11]. The bacteria usually are unable of developing resistance against Ag-NPs, because these nanomaterials can at the same time attack a broad range of targets in microorganisms such as proteins with thiol groups, cell walls and cell membranes. Some forms of silver have been demonstrated to be effective against burn infections, severe chronic osteomyelitis, urinary tract infections and central venous catheter infections[12]. Based on these results, many silver-based antimicrobial materials have become available and several others are under development in research laboratories[13, 14]. Feng *et al.* treated these bacteria with $AgNO_3$ and studied the effects on cell morphology using combined electron microscopy (TEM and SEM) and X-ray microanalysis[15]. *E. coli* and *S. aureus* underwent similar morphological changes after silver ion treatment characterized by a cytoplasm membrane detachment from cell walls and the appearance of an electron-light region in the center of the cells, which contained condensed deoxyribonucleic acid (DNA) molecules probably formed to protect DNA from injuries mediated by the silver ions.

Licorice, *Glycyrrhiza glabra* L. (Fabaceae), is considered as one of the oldest and most widely used herbs around the world[16]. Licorice has been traditionally used in herbal medicines for its emollient, antitussive and gastroprotective properties[17]. A number of bioactive compounds in *G. glabra* have been reported *viz.* glycyrrhizin, glycyrrhetic acid, liquiritin, liquiritin apioside, isoliquiritin and glabridin[18-20]. Glabridin, the major flavonoid of *G. glabra* possesses a wide range of activities viz. antioxidant[21], anti-helicobacter pyloric activity[22], estrogen-like activity[23], antinephritic and radical scavenging activities[24] as well as inhibition of serotonin re-uptake[25]. The aqueous extracts of liquorice contain 5-10% of a sweet, white, crystalline diglucuronide known as glycyrrhizin (Nirmala and Selvaraj). Recently, glabridin was known as a potent anti-mycobacterial and also effective against several human cancer cell lines. In another study licorice hydrophobic flavonoids (containing glabridin 1.2% (w/w) exhibited abdominal fat lowering and hypoglycemic effects in obese diabetic KK-Ay mice[26]. A rapid, precise and sensitive analytical method is needed for the determination of glabridin in *G. glabra* as well as in herbal products. Reported HPLC methods[27-30] are tedious and time consuming. In this study, we developed and validated a rapid analytical method for quantitation of glabridin using HPLC.

This study is based on the synthesis of silver nanoparticles, reducing the silver ions present in the solution of silver nitrate by the cell free aqueous extract of *Glycirrhiza glabra* root. Furthermore these biologically synthesized nanoparticles were found to produce broad spectrum antimicrobial activity.

MATERIALS AND METHODS

Preparation of plant material

Glycirrhiza glabra root was collected from local surroundings of Madurai region. Roots were used for the extraction of active components. The plant materials were shade dried at room temperature and powdered. These powdered samples were stored in an air tight container.

Qualitative phytochemical analysis

The dried plant material was successively extracted with chloroform, methanol, water and kept in shaker for 2 days. The solvent was evaporated using rotary evaporator under reduced pressure at 37° C. The plant extract was subjected to phytochemical analysis by the method described by Harborne[31]. The extract was tested for the presence of bioactive compounds like alkaloid, flavonoid, glycosides, phenol, saponin, steroid, tannin and terpenoids.

Synthesis of silver nanoparticles

Aqueous solution (1 mM) of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 5 ml of the extract was added into 95 ml of aqueous solution of 1 mM silver nitrate for reduction into Ag+ ions. In a typical synthesis of silver (Ag) nanoparticles the extract (1.5 ml) was added to 30 ml of 10^{-3} M $AgNO_3$ aqueous solution in a 250-ml Erlenmeyer flask and heated on water bath at 75 °C for 60 min. Reduction of silver nitrate to silver ions was confirmed by the color change from colorless to brown. The formation of silver nanoparticles was also confirmed by spectrophotometric determination. The obtained pellet was redispersed in deionized water and the centrifugation process was repeated two to three times to wash off any absorbed substances on the surface of the silver nanoparticles[32].

Microorganisms

Bacteria such as *Escherichia coli*, *Bacillus cereus*, *Klebsiella pneumonia, Klebsiella terrigena, Mycobacterium mucilaginosus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Fungal organisms such as *Fusarium oxysporum*, *Penicillium* and *Aspergillus niger* were selected for the assay. The bacterial cultures were maintained on nutrient agar slants at 4°C and the fungal cultures were maintained on potato dextrose broth at 25°C.

Preparation of inoculum

The bacterial cultures were inoculated into nutrient broth and incubated for 24h at 37°C. The growth was compared with 0.5 McFarland; the turbidity of the medium indicates the growth of organisms, while the fungal cultures were inoculated into potato dextrose broth and allowed to incubate at 25°C for 48 h[33].

Antimicrobial assay of Silver Nanoparticles

The silver nanoparticles synthesized from *G. glabra* were tested for antimicrobial activity by well-diffusion method against pathogenic microorganisms. Standard well agar diffusion method was carried out to detect the activity of Ag-Nps against the microbial isolate according to Cheesbrough[34]. For antimicrobial activities of the compounds, wells were made in plates containing nutrient agar medium seeded with 100 µl of 24 h of each microbial isolate. The wells were loaded with plant extract, silver nitrate and plant mediated synthesized silver nanoparticle was loaded using a micropipette. From each solution, that contains both $Agno_3$, *G. glabra* extracts, synthesized silver nitrate was placed in separate wells. The plates were left in refrigerator for 2 h then, incubated at 37°C for 24 h. The diameter of inhibition zones was measured and tabulated.

UV-Vis spectroscopy

The reaction mixture were observed visually for any colour change and one ml of reaction mixture were withdrawn at different time intervals by diluting a small aliquot (100µl) of the sample 10-fold in deionized water for analysis of surface plasmon resonance of silver nanoparticles. The reduction of pure Ag+ ions was monitored by measuring using a UV-Vis spectrophotometer (Shimadzu 1601 model, Japan) at the resolution of 1 nm in range of 200 to 800 nm.

FT-IR analysis

The surface groups of the nanoparticles were qualitatively confirmed by using FTIR spectroscopy[35] with spectra recorded by a Perkin-Elmer Spectrum 2000 FTIR spectrophotometer. FT-IR analysis was performed using Shimadzu FT-IR model number 8400. Approximately three mg of lyophilized leaves extract under study was mixed with 300 mg of dried KBr, crushed well in mortar and pestle to prepare thin pellet for analysis. Same procedure was performed for synthesized AgNPs using root extract. 16 scans per sample were taken in range of 400-4000 cm^{-1}.

Scanning electron microscopy (SEM)

The structure and composition of freeze-dried purified silver particles were analyzed by using a 10-kV ultra-high resolution scanning electron microscope. A drop of aqueous solution containing purified silver nanomaterials obtained after repetitive centrifugation was sputter coated on carbon coated copper grids and the images of nanoparticles were studied using FEI QUANTA-200 SEM.

RESULTS AND DISCUSSION

Table 1: Phytochemical analysis of *G. glabra*

Phytochemicals	Chloroform	Methanol	Ethanol	Water
Alkaloid	+	+	-	-
Flavanoid	+	+	+	+
Saponin	+	+	+	+
Tannin	+	+	-	-
Phenol	-	-	-	-
Glycosides	+	+	-	-
Terpenoid	+	+	+	-
Steroid	+	+	+	+

Phytochemical constituents of *G. glabra* were represented in Table: 1. Methanol and chloroform indicates the presence of active constituents except phenol. Polarity, structural stability and mass transfer parameters such as diffusibility, coefficient, molecular stability and concentration gradient might attribute to the presence of more components in chloroform[35]. Ethanol and water extract indicates the presence of flavonoid, terpenoid, saponin and steroid.

Visual colour change of silver nanoparticle

The UV-VIS Spectral analysis of the green synthesised nanoparticles was observed and a sharp peak at 240nm indicates the formation of silver nanoparticles. Yellowish brown colour is formed in aqueous solution is the visual indication of the synthesis of AgNPs. The relative percentage of scatter or absorption from the measured extinction spectrum depends on the size, shape, composition and aggregation of sample. Scattering contribution increases as the particles aggregate to a greater extent. The optical properties of silver nanoparticles change when particles aggregate and the conduction electrons near each particle surface become delocalized and are shared amongst neighbouring particles.

Uv-vis analysis of synthesized silver nanoparticle

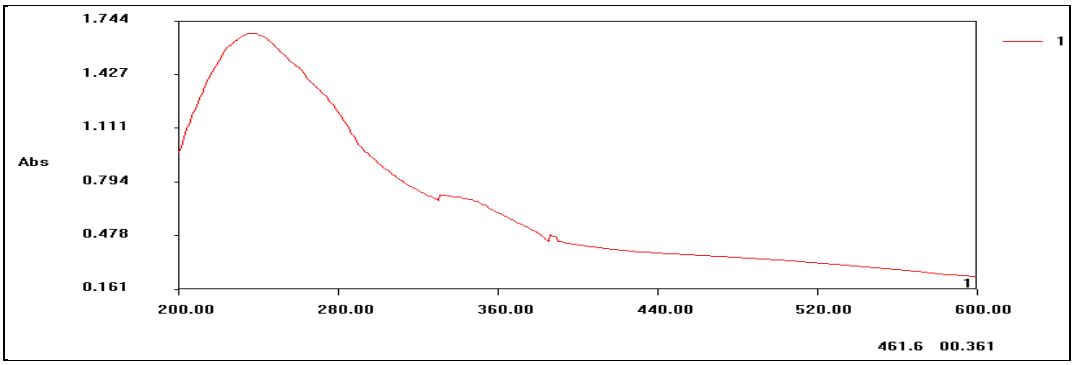


Figure 1: Uv-vis analysis of synthesized silver nanoparticle at 75° C

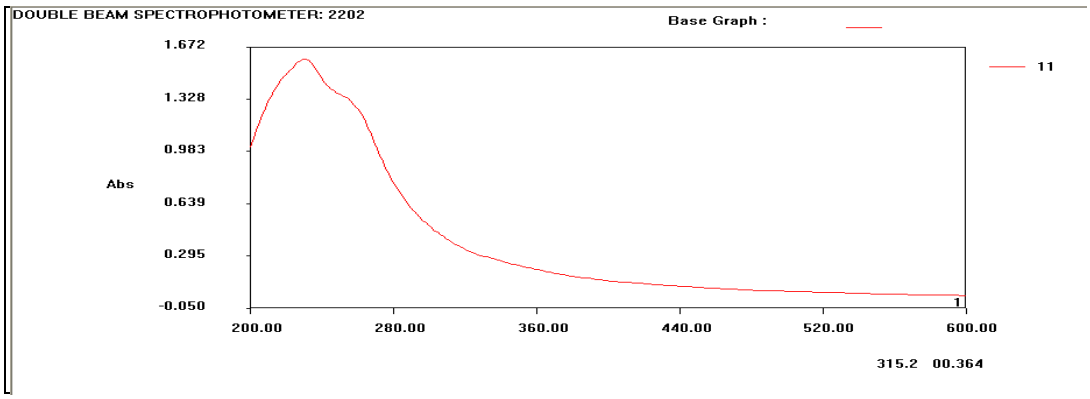


Figure 2: Uv-vis analysis of synthesized silver nanoparticle at 60 min

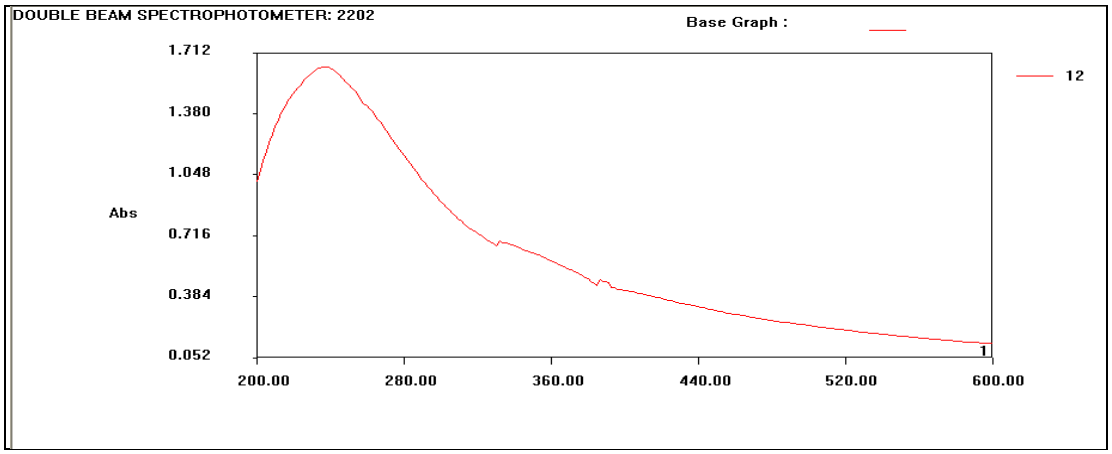


Figure 3: Uv- vis analysis of synthesized silver nanoparticles at pH-4

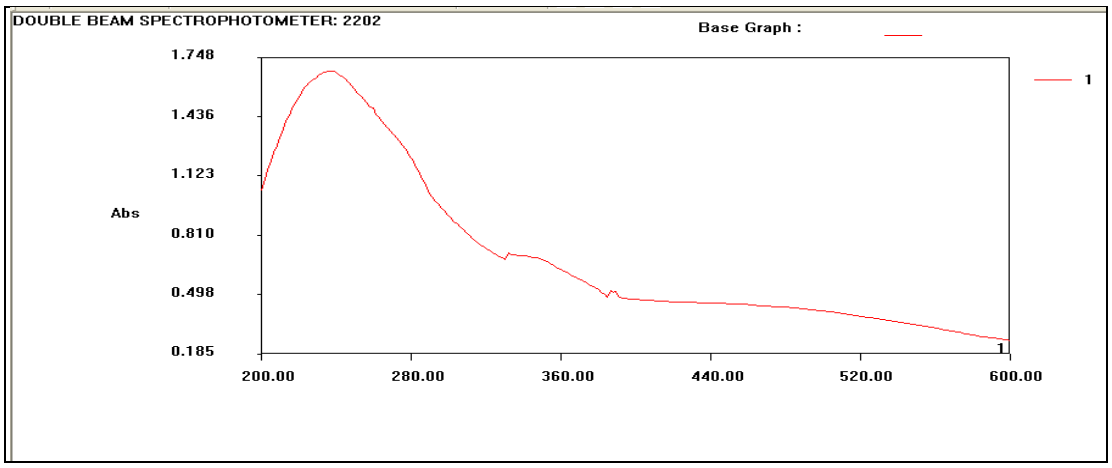


Figure 4: Uv- vis analysis of synthesized silver nanoparticles at pH-8

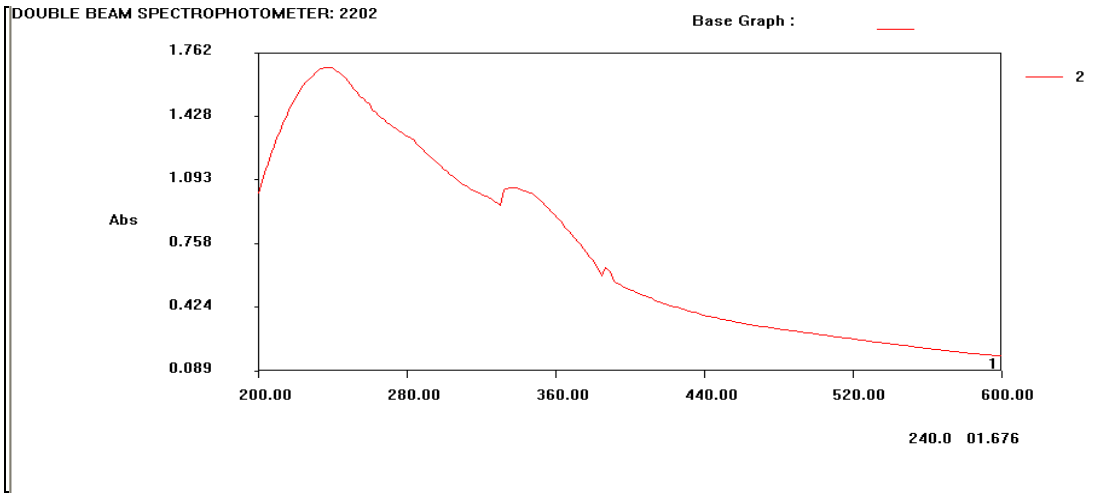


Figure 5: Uv- vis analysis of synthesized silver nanoparticle at 1mM

Optimization parameters for synthesis of silver nanoparticles

Primary confirmation for formation of nanoparticles is carried out by UV-Visible spectroscopic technique[36]. In the present study Uv- vis analysis of synthesized Agnps using different factors were represented in the Figure: 1-5. In this study optimized parameters includes temperature, pH, concentration of silver nitrate and time for synthesis of silver nanoparticles. Rai & Yadav reported that several factors that affect the reduction process of silver ions into the AgNPs[37].

Ambient temperature is one of the most stimulating aspects of AgNPs synthesis. However, the size and shape of nanoparticle is determined by the temperature of the reaction mixture which is a precarious factor. Song and Kim assessed the biosynthesis of AgNPs at different temperatures (25, 55 and 95°C) [38]. In the existing study optimum yield of silver nanoparticle is obtained at 75°C.

They reported that the gradual increase in temperature of reaction mixture results in an increase in the rate of biosynthesis in addition to the transformation of silver ions to AgNPs. The final transformation of silver ions was 60% at 25°C which increases to almost 100% at 55°C. However, the size of AgNPs decreases with increase in temperature (25°C-95°C) from 50 nm to 16nm.

The next factor was the incubation time required for the completion of reaction. As the duration of reaction increases, more silver nanoparticles are formed. Optimum period is required for formation of larger particle sizes. The optimum time required for the completion of reaction from our study was 60 min.

The pH of the reaction mixture is a vital factor that is considered to affect the size and shape of nanoparticles. In previous study AgNPs were synthesized using *Cinnamomum zeylanicum* leaf extract at different pH values ranging from 1 to 11[39]. They reported that the AgNPs of large size having ellipsoidal shape were observed at lower pH, while AgNPs with small size having spherical shape were observed at higher pH[40]. The aqueous solution of AgNPs exhibit different SPR behavior at different pH values that was enlightened in terms of size and size distribution of AgNPs[41].

The change in concentration of the plant extract also affects the synthesis of AgNPs[38]. Bar *et al*., studied the effect of different concentrations of latex and $AgNO_3$ on synthesis of AgNPs[42]. In the present study maximum yield is obtained with 1 mM silver nitrate solution. Besides that, the ratio of silver nitrate solution (1 mM) and the leaves extract was altered to investigate the optimum composition to maximize the yield of silver nanoparticles. Effect of different concentrations of $AgNO_3$ (1, 3 and 5 mM) was also studied and it was observed that the intensity of surface plasmon band increases with the increase in the concentration of $AgNO_3$. The results also revealed that an increase in the intensity of SPR also resulted in the increase of concentration of AgNPs. Consequently, different factors direct the synthesis of AgNPs by changing their size and shape.

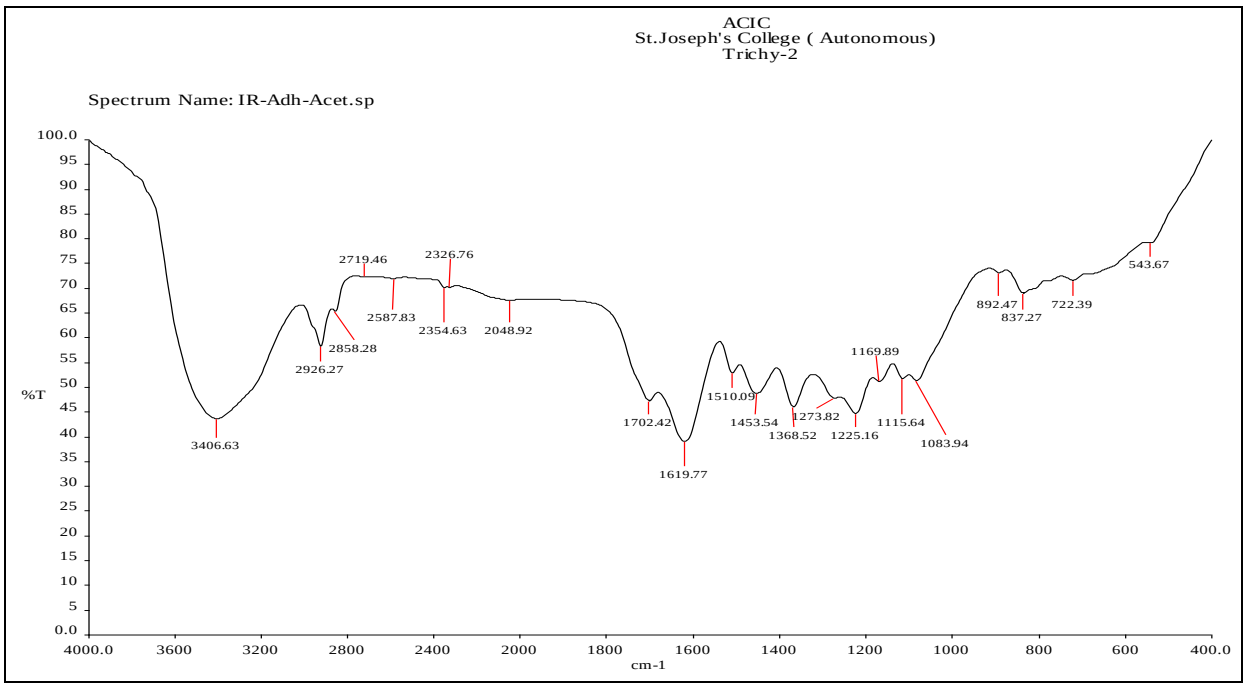


Figure 6: FTIR analysis of synthesised Agnp using *G. glabra*

FTIR analyses of synthesized Agnp using G. glabra were represented in Figure: 6. The absorption band of glycirrhizin at 3406^{cm-1} indicates the presence of OH stretch. The peak at 1619 $^{cm-1}$ indicates the presence of primay amine NH bend, In the previous study the absorption peaks of respective functional groups (amide and amino) indicate the presence of stabilized protein molecules[43,44]. In existing study the wavenumber at 1368^{cm-1} indicates the presence of phenol group. FTIR analysis revealed that the phenolic compounds like the ophylline and caffeine existing in the *Camellia sinensis* extract are accountable for the stabilization of AgNPs[45, 46]. The peaks at 1510 & 1453 $^{cm-1}$ indicates the presence of aromatic ring stretch, 1273^{cm-1} indicates the presence of aromatic primary amine. The wavenumber obtained at 1115.64^{cm-1}, 1083.94^{cm-1} indicates the presence of ether groups, whereas 1169^{cm-1} indicates the presence of tertiary amine, C-N stretch. The peak at 722^{cm-1} indicates the presence of alcohol group. FTIR spectral results revealed that most of the bands were representative of flavonoids and terpenoids and vibrational bands corresponding to bonds such as –C=C, –C–O–C, –C=O, –C–O and –C–N were derived from the plant metabolites like thiamine, flavonoids and terpenoids present in *Glycyrrhiza glabra* roots. The functional assignments indicate that these biologically active compounds act as reducing and stabilizing agents for the AgNPs[47]. Hence, from FTIR analysis it may be assumed that these

biomolecules are accountable for capping and proficient stabilization of AgNPs[48].

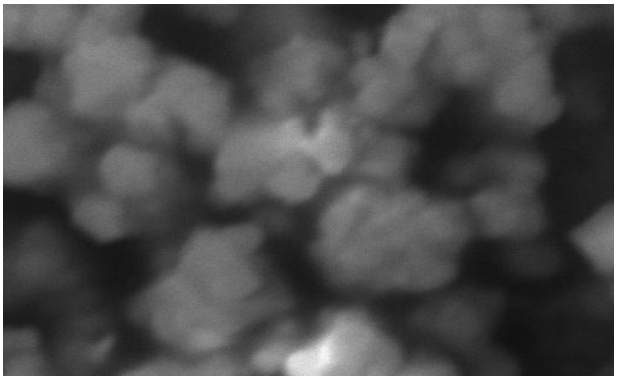
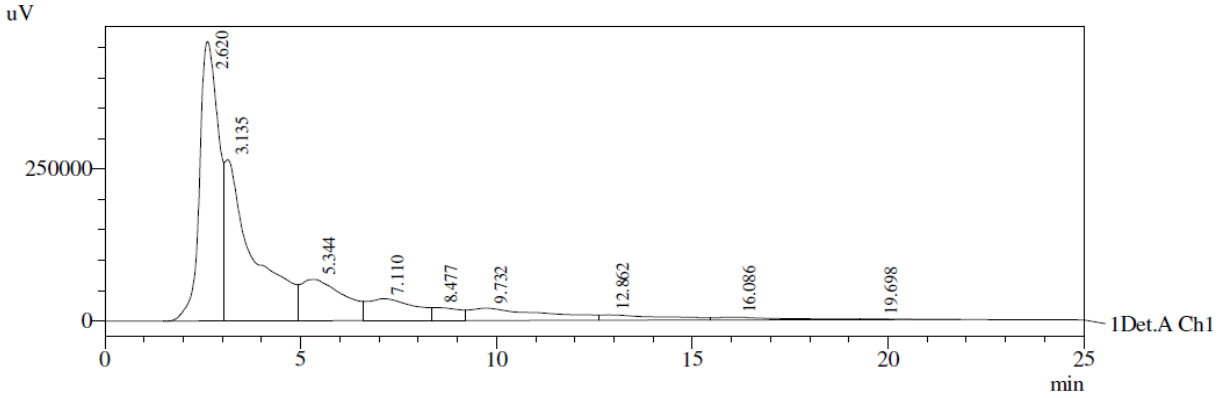


Figure 7: SEM analysis of synthesized silver nanoparticle using *G. glabra*

This study depicts that SEM analysis of synthesized Agnp were of sphere shape were represented in Figure: 7. Precise determination of AgNPs size, size distributions and size dimensions is vital not only for characterization of important size-dependent properties but also for many other imperative scientific applications. TEM and SEM are the universally employed techniques for measuring the size of nanoparticles. This is due to their ability of providing all information directly related to the morphology of metallic nanoparticles[49].



1 Det.A Ch1 / 254nm

PeakTable

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.620	15764231	459490	36.633	51.965
2	3.135	13575418	265330	31.547	30.007
3	5.344	5215121	67858	12.119	7.674
4	7.110	3055421	35950	7.100	4.066
5	8.477	985669	21199	2.290	2.397
6	9.732	2744056	20142	6.377	2.278
7	12.862	998146	8660	2.319	0.979
8	16.086	594705	4313	1.382	0.488
9	19.698	100212	1293	0.233	0.146
Total		43032977	884234	100.000	100.000

Figure 8: Hplc analysis of *G.glabra*

Hplc analysis of G. glabra were represented in Figure: 8. The described HPLC method has the advantage of simplicity, precision, accuracy and sensitivity for the analysis of glabridin in *G. glabra* root. Glabridin is a species specific and a minor compound of *G. glabra* [5]. Retention time at 2.2 indicates the presence of 36% liquiritin, liquiritigenin glycoside(31.5%), glycyrrhizin(12%), glycyrrhetin glycoside(2.2%), glabridin (7.1%), glabrene(6.3%). This method was validated for linearity and precision in the studied concentration range. It can be concluded that because of the simple extraction procedure, high precision, accuracy and a short run time, this method may be useful for screening of glabridin in raw material as well as in herbal products.

Antimicrobial activity of *G. glabra* were represented in Table: 2. Maximum inhibitory effect was observed in acetone extract against *B. cereus* (23 mm) and *E. coli* (22.5 mm). Ethyl acetate extract remained resistant towards *S. aureus*(22.5 mm), *K. terrigena*(22 mm), *B. cereus* (21.6 mm), *M. mucilaginosus*(21.6 mm), *E. coli*(21.5 mm). Ethanol extract acquired maximum inhibiton against *K. terrigena*(21.5 mm), *E. coli*(20.6 mm), *K. pneumonia*(20.5 mm). Chloroform extract possess moderate zone of inhibition against *K. terrigena*(16 mm), *M. mucilaginosus*(15.6 mm), *P. aeuroginosa*(15.3 mm). Petroleum ether extract remained sensitive towards *M. mucilaginosus*(14.3 mm), *B. cereus*(14.1 mm), *K. pneumonia*(13.5 mm). Antifungal activity was found to be maximum in acetone extract against *Penicillium*(21 mm), *A. niger*(20.5 mm), *F. oxysporum*(20.1 mm). Minimum inhibitory effect was observed in petroleum ether extract against *A. niger*(13.5 mm), *Penicillium*(13 mm).

Table 2: Antimicrobial activity of *Glycirrhiza glabra*

S.no	Microorganism	*Glycirrhiza glabra*				
		Acetone	Chloroform	Ethanol	Ethyl acetate	Petroleum ether
1.	*Bacillus cereus*	23±0	20.5±0.5	19±0	21.6±0.2	14.1±0.2
2.	*Klebsiella pneumonia*	20.8±0.2	13.6±0.2	20.5±0	21.5±0.8	13.5±0
3.	*Pseudomonas aeuroginosa*	19.5±0	15.3±0.2	18.5±0.5	20.6±0.5	14.5±0
4.	*Staphylococcus aureus*	19.1±0.2	18.6±0.7	19.5±0.5	22.5±0.5	18.1±0.2
5.	*Escherichia coli*	22.5±0.2	17±0	20.6±0.5	21.5±0.5	16.3±0.2
6.	*Mycobacterium mucliaginosus*	20.5±0	15.6±0.5	20.3±0.5	21.6±0.2	14.3±0.2
7.	*Klebsiella terrigena*	19.6±0.2	16±0	21.5±0.5	22±0	15±0
8.	*Fusarium oxysporum*	20.1±0.2	14.5±0	17.8±0.2	17.3±0.2	18.3±0.5
9.	*Penicillium*	21±0	14.3±0.2	17.5±0	20.5±0.5	13±0
10.	*Aspergillus niger*	20.5±0	19.3±0.2	16±0	17.8±0.2	13.5±0

*values are mean of ± S.D, n=3

Table 3: Antimicrobial activity of synthesized silver nanoparticle

Microorganism	Plant samples used in the study Zone of inhibition in mm			
	Glycirrhiza glabra			
	100	200	300	400
Bacillus sp.	18.3±0.2	20.5±0	22.3±0.2	24±0
Escherichia coli	18±0	19.1±0.2	20.5±0	22.1±0.2
Mycobacterium mucilaginosus	15.1±0.2	16.5±0	18.3±0.2	20.5±0
Klebsiella terrigena	16.3±0.2	18.3±0.2	19±0	21.3±0.2
Pseudomonas aeruoginosa	17.5±0	18.4±0.1	20.1±0.5	22±0
Shigella	14.1±0.2	16.3±0.2	17±0	19.3±0.2
Staphylococcus epidermis	18±0	19.3±0.2	21.3±0.2	23±0
Fusarium oxysporum	15±0	17.1±0.2	19.1±0.2	20.5±0.5
Penicillium	14.5±0	16±0	17.3±0.2	19.5±0
Aspergillus niger	13.3±0.2	15.1±0.2	16.5±0.5	18.3±0.3

*values are mean of ± S.D, n=3

Microbicidal activities of synthesized silver nanoparticle were represented in Table: 3. to investigate the antibacterial properties of AgNPs, Kim *et al*. performed an experiment by using a model of both Gram-positive (*S. aureus*) and Gram negative (*E. coli*) bacteria[50]. 400 µl of synthesized Agnps possess maximum zone of inhibition against *Bacillus sp*. (24 mm), *S. epidermis* (23 mm), *P. aeruoginosa*(22 mm), *K. terrigena* (21.3 mm). 300 µl remained resistant towards *Bacillus sp*. (22.3 mm), *S. epidermis* (21.3 mm), *E. coli* (20.5 mm). A recent study on *Escherichia coli* has shown that Ag-NPs react with cell walls and cytoplasmic membranes, resulting in pits in the cell wall of bacteria, and finally killing them[51]. Previous study reported that green synthesized titania (TiO_2) and silver nanocomposites (TANCs) can easily break the cell walls of *E. coli*[52]. Moderate zone of inhibition was observed in 200 µl against *K. terrigena*(18.3 mm), *M. mucilaginosus*(16.5 mm), *Shigella*(16.3 mm), whereas minimum inhibition was observed in 100µl against *M. mucilaginosus*(15.1 mm), *Shigella*(14.1 mm). In this study antifungal activity was found to be maximum in 400 µl of synthesized Agnps against *F. oxysporum*(20.5 mm), *Penicillium* (19.5 mm), *A. niger* (18.3 mm). Moderate inhibitory activity was observed in 200 µl against *A. niger* (15.1 mm). Minimum inhibition was acquired in 100 µl against *Penicillium* (14.5 mm) and *A. niger*(13.3 mm). A number of studies suggest that silver ions react with SH groups of proteins [53, 54] and play an essential role in bacterial inactivation[55]. Silver ions and silver nanoparticles also have inhibitory and lethal effects on bacterial species such as *E. coli*,[56, 57, 58, 59] *S. aureus*[56] and even yeast[56].

CONCLUSION

In this study biosynthesis of silver nanoparticles was done and *G. glabra* root extract was chosen as a reducing agent to generate Agnp. FTIR analysis showed the presence of different functional groups involved in capping the silver nanoparticles. Hplc analysis indicates the presence of bioactive constituents responsible for microbicidal activity. Our results suggests that the *G. glabra* root extract-capping AgNPs has potentials for medicinal applications.

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