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In-vitro Antioxidant and Antibacterial Activity of Green Synthesized Silver Nanoparticles using *Martynia annua* L. Leaves Extract

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ABSTRACT

Martynia annua, an herbaceous annual glandular hairy herb spread throughout the India has been used in traditional medicine and folklore for many diseases. Scientific investigation has revealed that this whole plant ethanomedically possess many pharmacological and therapeutic activities. This curative property makes it to the object of many researchers. The present study deals with the synthesis of silver nanoparticles (AgNPs) using the water leaf extract of *Martynia annua* by bioinspired route and examined its antimicrobial and antioxidant activities. The silver nanoparticles were synthesized using the aqueous leaf extracts of *Martynia annua*. The synthesized silver nanoparticles were confirmed by UV Visible spectrophotometer at 454 nm. FTIR analysis of synthesized nanoparticles revealed that the presence of phytochemical compounds in the plant extract responsible for the formation, capping and stabilization of nanoparticles. Further characterization of the biosynthesized silver nanoparticle was performed using sscanning electron microscopy, particle size analyzer, energy dispersive X-ray spectroscopy and zeta ppotential for the analysis of the size, shape, average size, electric charge, elemental composition and stability of synthesized nanoparticles. The shape and average size of the synthesized nanoparticles were showed at spherical in the size range 60.12 nm. The presence of elements, silver (3 KeV) was confirmed by EDX analysis. Zeta potential showed that synthesized nanoparticles were found to be in negative charge in the value of -18.9 mV. Synthesized silver nanoparticles registered effective antibacterial activity against wound pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus sp*, *Bacillus subtilis* and *Enterococcus faecalis*. Synthesized nanoparticles also had shown a promising antioxidant activity.

***Key words*:** AgNPs, Antibacterial, Antioxidant, Cytotoxicity, *Martynia annua*

Nanotechnology is an emerging field of modern research with enormous applications such as antimicrobial, antioxidant, anti-inflammatory, antiviral, cytotoxic, anticancer and anti-HIV [15], [35], [39]. Wound healing management cope with restraining the wound site from microbial contagion and stimulating the tissue wound healing process. It involves three important phases such as cellular proliferation, remodeling phase and inflammation [2], [20]. Severe wound healing is always stimulated by high morbidity and longtime medical treatments of patients. Treating of wounds with time minimization for healing is always demand due to their chances of unwanted drawbacks. Conventional drug used for the wounds in long time may cause many side effects to kidney, heart, liver and to coma stage. Recently, synthesis of plant-based nanotechnology is practical and possible approach with their enhanced functionality and physcio chemical properties [27], [36].

Among the metal nanoparticles, AgNPs become very attractive and useful because of their excellent optical, electrical and biomedical properties [12]. Recently, researchers focused much attention for the synthesis of AgNPs due to their remarkable properties such as antimicrobial, wound healing, anticancer, tumour imaging, cosmetics, biosensor, photo-catalysis and drug delivery systems [14], [19], [26]. The synthesis of NPs using physical and chemical method requires high temperature, energy, pressure and toxic chemicals and its showed adverse effects in medical application [32].

Green chemistry employs of a set of principles that will restrain the use and generation of precarous substances during the assembling and implementation of chemical products. The various phyto-chemical metabolites present in the extract is responsible for the formation of metal ions to metal nanoparticles [4]. The plant *M. annua* belongs to the family *Martyniaceae*, and is commonly known as the Cat's claw or Devil's claw and bichu. The biological active molecules including carbohydrates, phenols, anthocyanins, flavanoids and tannins in the leaves and fruits of this plant are used for the

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treatment of various diseases like antibacterial, antifungal, antiseptic, antiepileptic and wound healing activities [17].

Linear application of plant extracts as ointments on wounds are not much effectual hence employing the silver nanoparticles from natural medicinal plant extract for testing its efficacy over the later on wound healing [6], [37]. In a view of above, the present research explore the facile synthesis, characterization, antimicrobial and wound healing potential of AgNPs from *M. annua* leaf extract by green chemistry approach.

MATERIALS AND METHODS

Collection of plant material and chemicals

The fresh leaves of *Martynia annua* were collected in and around place of Kerala, India. The collected plant material was identified at Botanical Survey of India (Reference number: BSI/SRC/5/23/2018/Tech/2339), southern region, Coimbatore. Chemicals such as silver nitrate, MTT, DPPH, sulfuric acid, sodium phosphate, ammonium molybdate, acetate buffer, TPTZ, HCL, $FeCl_3$ and media viz., muller hinton agar, nutrient broth were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Wound pathogenic cultures of *Eschericia coli*, *Staphylococcus aureus*, *Streptococcus sp*, *Bacillus subtilis* and *Enterococcus faecalis* were obtained from Microbiological laboratory, Coimbatore, India.

Preparation and extraction of plant powder for phytochemical screening

Fresh leaves were collected from the study plant, thoroughly washed with tap water, shade dried and ground into a coarse powder which is used for further investigations. Extraction was carried out using water as solvent. Coarsely powdered plant material were extracted using water through Soxhlet apparatus. The collected extracts were used for preliminary screening of phytochemicals. The obtained crude extract was stored at 4°C for further use.

Preliminary qualitative analysis of phytochemicals

The condensed extracts were used for preliminary screening of phytochemicals such as alkaloids, glycosides, saponins, phenolics tannins, phytosterols, carbohydrates, proteins, aminoacids, flavanoids, quinones and terpenoids. The presence of phytochemicals from aqueous extracts was qualitatively determined by following the standard methods of (Brindha 1981), (Harborne 1998), (Lala 1993), (Kokate *et al*. 2004), (Edeoga *et al*. 2005), (Khandelwal 2008) [11], [16], [21], [22], [25].

Synthesis and characterization of silver nanoparticles

For biosynthesis of silver nanoparticles, 90 ml of aqueous solution (1 mM) of silver nitrate ($AgNO_3$) was mixed with 10 ml of aqueous leaves extract. The solution was kept in the stirrer for 60min and reduction of silver nitrate to silver ions was confirmed by the color change from colorless to brown.

The formation of AgNPs was primarily confirmed by Shimadzu 1800 UV-vis spectrophotometer in the wavelength range of 200-800nm. The presence of different functional groups was analyzed using FT-IR spectrophotometer (Shimadzu, 8400, Japan) using KBr. The average particle size, surface morphology and elemental composition of synthesized AgNPs were studied by Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) (SIGMA model, CASL ZEISS, German) operating at an accelerating voltage of 3.00 KV. The particle size distribution of AgNPs was evaluated using dynamic light scattering measurements (DLS) and zeta potential analysis was conducted with Malvern zetasizer neroseries compact scattering spectrometer (Malvern Instruments Ltd., Malvern, UK).

Determination of antioxidant activities

DPPH free radical scavenging activity

The free radical scavenging activity of the synthesized AgNP was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH [28]. Ascorbic acid was used as standard. Percentage inhibition of DPPH free radical was calculated based on the blank reading, which contained DPPH and distilled water without extract using the following equation:

$$DPPH Scavenging\ activity(\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

The antioxidant activity of the AgNP was expressed as IC50. The IC50 value was defined as the concentration (in µg/ml) of AgNP that inhibits the formation of DPPH radicals by 50%.

Ferric reducing antioxidant power

The reducing capacity of the synthesized AgNP was estimated spectrophotometrically following the method of Iris FF Benzie and John J Strain [18]. All the determinations were performed in triplicates and the values are expressed as ascorbic acid equivalents in micro per mg of AgNP.

Determination of Total antioxidant capacity

Total antioxidant capacity was measured according to the method prescribed by Prieto et al., 1999 with slight modifications. The samples of unknown composition, water-soluble antioxidant capacities are expressed as equivalents of ascorbic acid. The experiment was conducted in triplicate and values are expressed as ascorbic acid equivalents in micro per mg of AgNP.

Total flavanoid content

Total flavanoid content of plant extract was determined by using aluminum chloride colorimetric assay [29]. The standard curve of quercetin was made and the total flavonoids content was expressed as milligrams of quercetin equivalents per 100 µm of extracts.

Antibacterial activity

Minimum inhibitory concentration

MIC is defined as the lowest concentration of the nanoparticles that inhibit the growth of the test cultures. The MIC of synthesized AgNPs was determined by following the standard protocol of Arasu *et al*. [5]. *Eschericia coli*, *Staphylococcus aureus*, *Streptococcus sp*, *Bacillus subtilis* and *Enterococcus faecalis* were used as test organism. Antibiotic amoxicillin was used as control. The experiment was repeated thrice to confirm the antibacterial activity of AgNPs.

Statistical analysis

The data of all parameters were analyzed using Minitab 16 statistical software. Statistical analysis was performed by using One Way Analysis of Variance (ANOVA) followed by Dunnett's test. The groups will be compared with respective control groups. The values were expressed as mean ± Standard Error Mean (S.E.M). *P value < 0.05 was considered as significant.

RESULTS AND DISCUSSION

The aqueous leaf extract of *Martynia annua* were subjected to preliminary phytochemical analysis (Table 1). The results revealed that the presence of secondary metabolites such as alkaloid, glycosides, saponins, phenolic compounds, tannins, carbohydrates, flavanoids, proteins, terpenoids and steroids while the compounds like the phytosterols, aminoacids and quinines were absent in aqueous extract. The preliminary screening analysis is helpful in the detection of bioactive compounds and lead to the discovery and development of novel drugs [16].

Table 1 Preliminary phytochemical profile of aqueous leaf extract of *M. annua*

Phyto constituents	Tests	Existence
Alkaloids	Mayer's test	+
Glycosides	Borntrager's test	+
Saponins	Froth forming test	+
Phenolic compounds	Lead acetate test	+
Tannins	Fecl3 test	+
Phytosterols	Libermann Buchard test	-
Carbohydrates	Fehilings test	+
Proteins	Biurret test	+
Amino acids	Barfoed's Test	-
Flavanoids	Alkaline test	+
Quinones	Quinone test	-
Terpenoids	Terpenoids test	-

'+' - Presence '-' – Absence
Values are mean ±S.E.M (n=3)

Fig 1 Synthesis of silver nanoparticles using aqueous leaf extract of *Martynia annua*

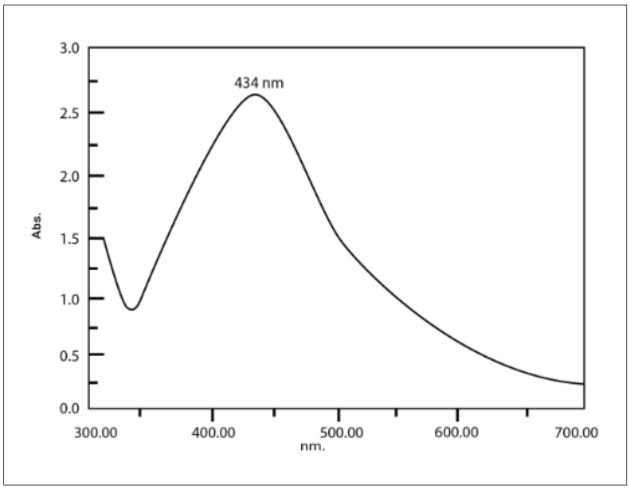

Fig 2 UV-visible spectrum of biosynthesized silver nanoparticles using aqueous leaf extract of *M. annua* at different time intervals

Preliminary confirmation and characterization of biosynthesized silver nanoparticles

Reductions of Ag+ ions into silver nanoparticles with the addition of *Martynia annua* leaf extract were observed based on colour change. It was observed that the color of the solution turned from yellow to bright yellow then to dark brown which indicated the formation of AgNPs was shown in (Fig 1). The colour change of the solution is due to the surface plasma resonance phenomenon on the surface of nanoparticles [8], [9], [24]. The formation and stability of reduced silver nanoparticles was further monitored by UV-vis spectrophotometer analysis at various time intervals was shown in (Fig 2). The curve showed increased absorbance in various time intervals and the peaks were noticed at 434 nm corresponding to the surface plasma resonance of silver nanoparticles.

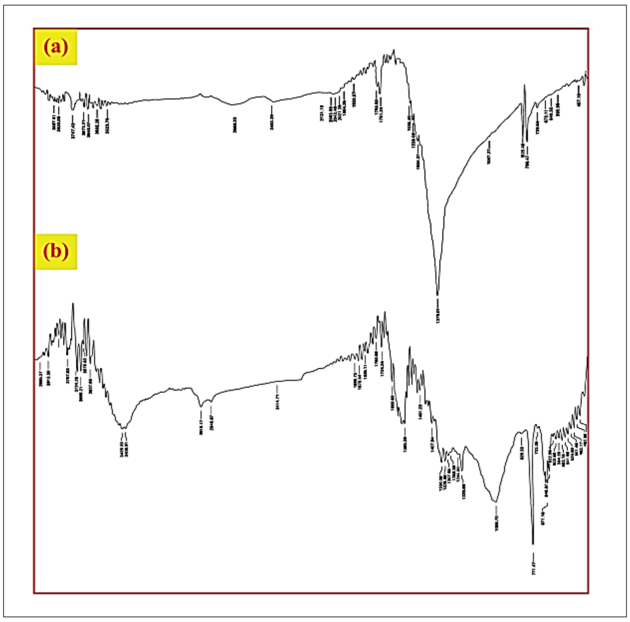

Fig 3 FT-IR analysis of biosynthesized silver nanoparticles using aqueous leaf extract of Martynia annua. (a) FT-IR of silver nitrate, (b) FT-IR analysis of silver nanoparticle

The functional groups of biomolecules involved in the reduction and efficient stabilization of the AgNPs were analyzed using FTIR spectra (Fig 3). FTIR spectra of $AgNO_3$ showed reveal absorption peaks at 2698.3 cm^{-1} is characteristic of O-H stretch, associated with the presence of hydroxylic acids, whereas the peaks at 1556.45 and 1504.37 cm^{-1} were shifted to 825.48 and 673.11 cm^{-1} is assigned to N-O stretch showed the presence of nitro compounds. The synthesized AgNPs shows absorption peaks at 3637 cm^{-1} is associated with OH stretch showed the presence of free hydroxyl which is also present in $AgNO_3$. The peak at 2916 and 2848 cm^{-1} are related with CH stretch showed the presence of alkanes. The peak at 1724 cm^{-1} and 1660 cm^{-1} is the characteristic of C=O stretch, showed the presence of aldehydes and unsaturated esters. The peak at 1660 and1585 cm^{-1} is associated with N-H bend showed the presence of primary amines. The peak at 1585 cm^{-1}, 1481 and 1407 cm^{-1} is related with C-C stretch showed the presence of aromatic carbon. The peak at 1328 and 1288 cm^{-1} is associated to C-N stretch showed the presence of aromatic amenes. The peak at 1307 and 1220 cm^{-1} belongs to C-H stretch showed the presence of alkyl halides. The peak at 829 and 671 cm^{-1} and 1481 cm^{-1} is associated with N-O stretch showed the presence of nitro compounds as in $AgNO_3$. FT-IR spectrum of the AgNPs provides the information about the functional groups involved in the reduction of the silver ions. The FT-IR analysis identify the minerals that capped on the silver nanoparticles and some possible biomolecules from *Martynia annua* leaf extract which reduce from Ag^+ to Ag^0. Further, this result showed that phytochemical constituents like alkaloids, tannins, saponins, glycosides, flavanoids, anthocyanins, amino acid, steroids and phenols might protect the AgNPs from aggregation and thereby retain them for long term stability [1].

X-Ray diffraction (XRD)

Crystalline state is an important criterion influencing solubility and stability behavior of the compounds. In general, an increase in saturation solubility and dissolution rate could be achieved by changing the crystalline state of the drug to amorphous.

A powder X-ray diffraction method is useful tool in identifying the physical nature of the particles. The diffraction pattern of silver nitrate exhibits the several sharp intense crystalline peaks expressed by numerous characteristic diffraction peaks of 2θ angle at 14.83º, 17.63º, 26.49º, 32.75º, 36.27º, 42.07º, 45.35º, 56.73º, 64.04° and 66.37º was shown in (Fig 4).

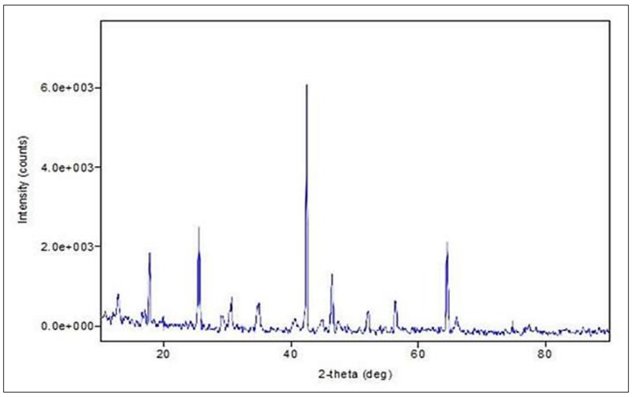

Fig 4 X-ray diffraction apttern of silver nitrate

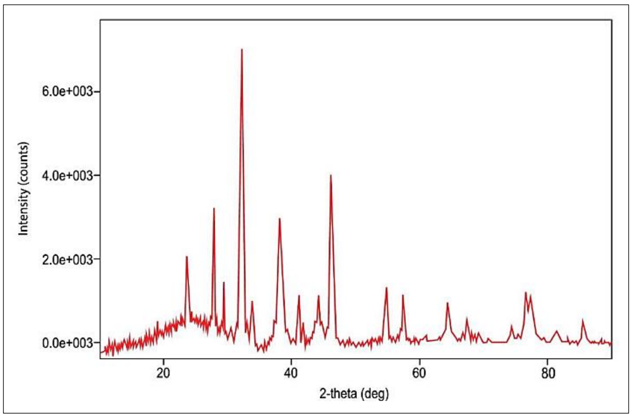

Fig 5 X-Ray diffraction pattern of the biosynthesized silver nanoparticle using aqueous leaf extract of *Martynia annua*

On the other hand powder X-ray diffraction pattern of silver nanoparticle formulation also exhibit similar diffraction peaks in 2θ angle of 23.53°,27.76°, 29.41°, 32.18°, 33.75°, 38.05°, 41.12°, 44.13°, 46.15°, 54.74°, 57.41°, 64.40°, 67.37°, 74.36°, 76.63°, 81.51° and 85.58° was shown in (Fig 5). It indicates that the increase in the degree of crystalline as increased sharp distinctive peaks by the formation of silver nanoparticle from *Martynia annua* fresh leaf extract using silver nitrate. Previous studies revealed that tthese peaks might have been observed due to the various organic compounds present in the extract [34].

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analysis

The green synthesized silver nanoparticles were examined by scanning electron microscopy to study the morphology of synthesized nanoparticles. This SEM image revealed that the synthesized silver nanoparticles are small, well crystallized with smooth texture and globular in shape was shown in (Fig 6). Many studies have shown that size, shape, stability and physical properties of the metal nanoparticles depends on factors like experimental conditions including the preparation method and interaction of metal ions with reducing agent [13]. Energy dispersive X- ray spectroscopy (EDX) is an analytical technique used for elemental analysis and finds the percentage of chemicals composition on the surface of the synthesized nanoparticles. The result confirmed the presence of strong elemental signal of the silver at 3 KeV which is typical for the absorption of metallic silver nano-crystallites due to surface plasma resonance was shown in (Fig 7). The absorption peak of Ca, Na and Mg in the spectrum might be due to the involvement of phytoconstituents in capping and stabilizing of silver through the respective related groups [3], [30].

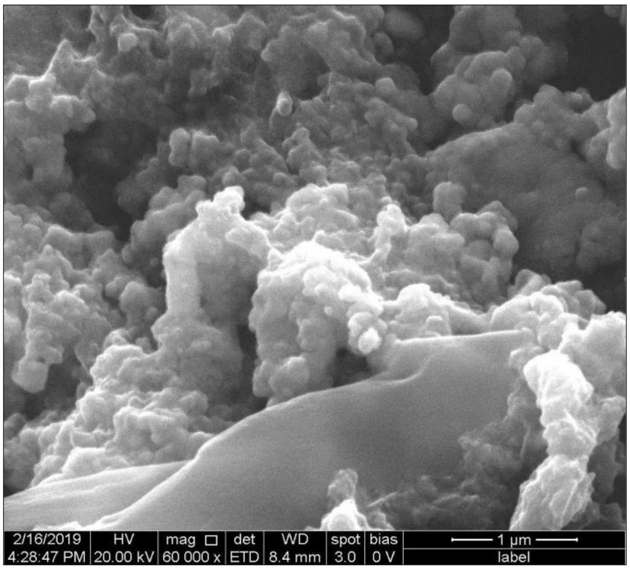

Fig 6 SEM images of biosynthesized silver nanoparticles using aqueous leaf extract of *Martynia annua*

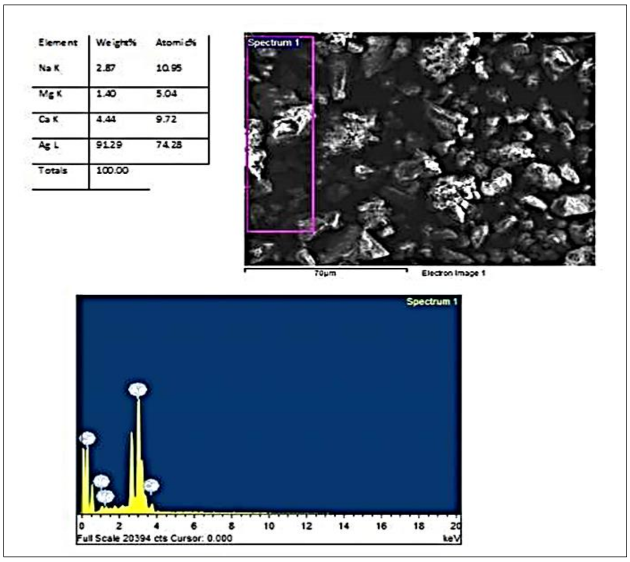

Fig 7 EDAX images showing the presence of silver nanoparticles and bio-organic components of *Martynia annua*

Particle size and Zeta potential analysis

Dynamic light scattering (DLS) analysis is used to determine the average particle size distribution profile of synthesized nanoparticles and capping agent enveloped the metallic particles along with the particle size of the metallic core [40]. The particle size distribution has most important characteristic affecting the *in-vitro* fate of nanoparticle. The particle size was measured by Malvern particle size analyzer. The size distribution of dynamic light scattering (DLS) indicates that the particle size of silver nanoparticle is 60.12 nm.

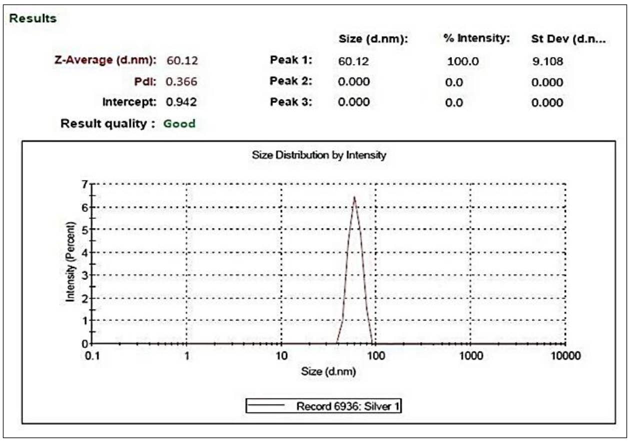

Fig 8 Particle size spectrum of biosynthesized nanoparticles using aqueous leaf extract of *Martynia annua*

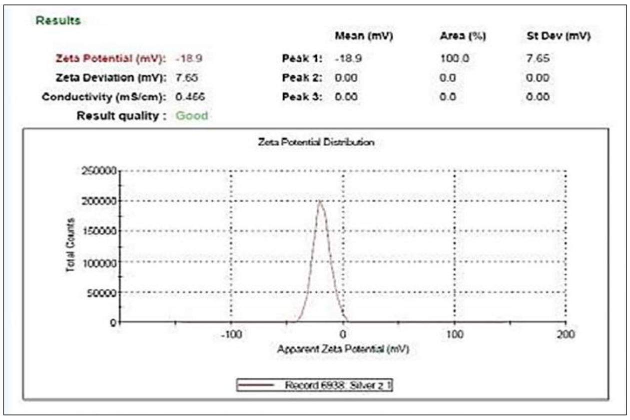

Fig 8 Zeta potential spectrum of biosynthesized nanoparticles using aqueous leaf extract of *Martynia annua*

The Zeta potential value of biosynthesized AgNPs shows a peak at -18.9 mV indicates that the surface of nanoparticles is negatively charged which shows the good stability of nanoparticles was shown in (Fig 8). It is evident that the AgNPs are polydispersed in nature, due to its high negative zeta potential; thus, the electrostatic repulsive force between them results in the prevention of agglomeration of the nanoparticles and is also very much helpful for long-term stability in the solution [23], [37]. The PDI value of biosynthesized silver nanoparticle is 0.366 which is associated with low particle size and least value of poly dispersity index, indicates good stability of nanoparticles. It is evident that the AgNPs are polydispersed in nature, due to its high negative zeta potential; thus, the electrostatic repulsive force between them results in the prevention of agglomeration of the nanoparticles and is also very much helpful for long-term stability in the solution.

DPPH free radical scavenging activity

The biosynthesized silver nanoparticles exhibit potent DPPH scavenging activity when compared with standard. It was observed that a significant decrease in the concentration of DPPH radical due to the scavenging ability of synthesized AgNPs was shown in (Table 2). IC_{50} value of ascorbic acid and synthesized AgNPs (concentration of sample required to scavenge 50% free radical) were found to be 22.69µg/ml and 63.11µg/ml respectively was shown in (Fig 9). The study revealed the synthesized AgNPs showed antioxidant activity due to capped phenolic compounds. Phenolic group facilitates the conversion of silver nitrate to AgNPs due to its electron donating ability [31].

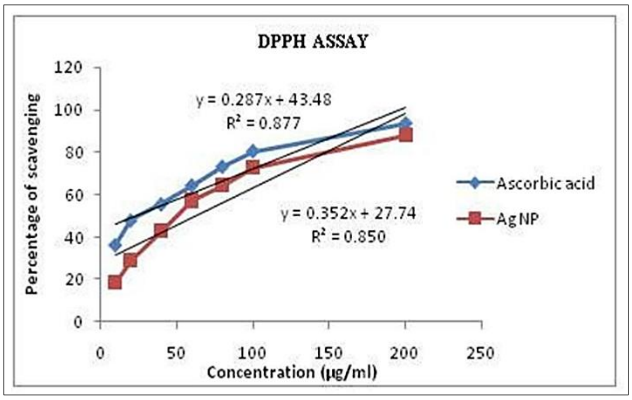

Fig 9 DPPH radical scavenging activity of silver nanoparticles

Table 2 DPPH scavenging activity of silver nanoparticles synthesized using aqueous leaf extract of *Martynia annua*

DPPH radical scavenging activity

S. No.	Concentration (µg/ml)	Percent of radical scavenging of ascorbic acid	Percent of radical scavenging of Ag NP
1	10	36.16 ± 0.27	18.67 ± 0.42
2	20	47.73 ± 0.18	29.14 ± 0.63
3	40	55.43 ± 0.73	42.94 ± 0.28
4	60	64.27 ± 0.58	57.34 ± 0.76
5	80	73.19 ± 0.39	64.62 ± 0.14
6	100	80.45 ± 0.63	73.17 ± 0.45
7	200	93.57 ± 0.23	88.17 ± 0.59
IC_{50} (µg/ml)	22.69 ± 0.43	63.11 ± 0.46	

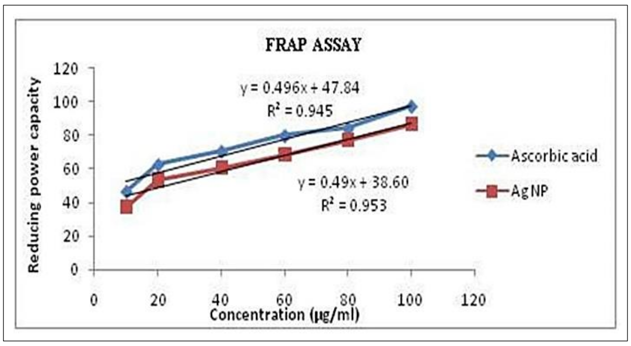

Fig 10 Ferric reducing antioxidant power activity of biosynthesized silver nanoparticles

Ferric reducing antioxidant power

The biosynthesized AgNPs possess effective reducing power when compared to the standard ascorbic acid. In order to identify reductive ability of AgNPs, transformation of the Fe^{3+} - Fe^{2+} was recorded in the presence of AgNPs using the method of Benzie and Strain [7]. IC_{50} value of ascorbic acid and synthesized AgNPs (concentration of sample required to scavenge 50% free radical) were found to be 4.3 µg/ml and 23.24µg/ml was shown in (Fig 10). The AgNPs exhibited significant reducing ability higher than that of standard ascorbic acid. This might be due to the presence of high phenolic concentration. Dipankar and Murugan [10] made similar observations in silver nanoparticles synthesised from *Iresine herbstii*.

Total antioxidant capacity

Total antioxidant capacity of biosynthesized AgNPs is expressed as number of equivalents of ascorbic acid. One mg of AgNPs and aqueous extracts contained 96.0 µg/ml and 94.0 µg/ml of ascorbic acid equivalents antioxidant capacity respectively.

Total flavanoid content

The total flavanoid contents were found to be 125.2 ± 6.38 mg (quercetin equivalent) per 100 µg of synthesized AgNPs.

Table 3 Ferric reducing antioxidant power (FRAP) activity of silver nanoparticles synthesized using aqueous leaf extract of *Martynia annua*

		FRAP activity	
S. No.	Concentration (µg/ml)	Percent of radical scavenging of Ascorbic acid	Percent of radical scavenging of Ag NP
1	10	46.49 ± 0.13	37.34 ± 0.43
2	20	62.52 ± 0.42	53.38 ± 0.25
3	40	70.49 ± 0.36	60.93 ± 0.63
4	60	79.84 ± 0.17	68.27 ± 0.54
5	80	84.16 ± 0.27	76.98 ± 0.25
6	100	97.43 ± 0.72	86.64 ± 0.72
IC_{50} (µg/ml)	4.348 ± 0.34	23.24 ± 0.47	

Values are mean ±S.E.M (n=3)

Minimum inhibitory concentration

The minimum inhibitory concentration produced by the commercial antibiotic, amoxicillin in different concentrations against *Escherichia* coli, *Staphylococcus aureus*, *Streptococcus sp*, *Bacillus subtilis* and *Enterococcus sp* was 5.61 µg/ml, 4.28 µg/ml, 1.14 µg/ml, 3.26 µg/ml and 59.14 µg/ml was shown in (Fig 11-12, Table 4). The minimum inhibitory concentration produced by the synthesized AgNPs against *Escherichia* coli, *Staphylococcus aureus*, *Streptococcus sp*, *Bacillus subtilis* and *Enterococcus sp* was 16.82 µg/ml, 19.55 µg/ml, 18.22 µg/ml, 11.44 µg/ml and 72.14 µg/ml. The result of the study revealed that the bioreduced AgNPs showed significant antibacterial activities when compared to the control.

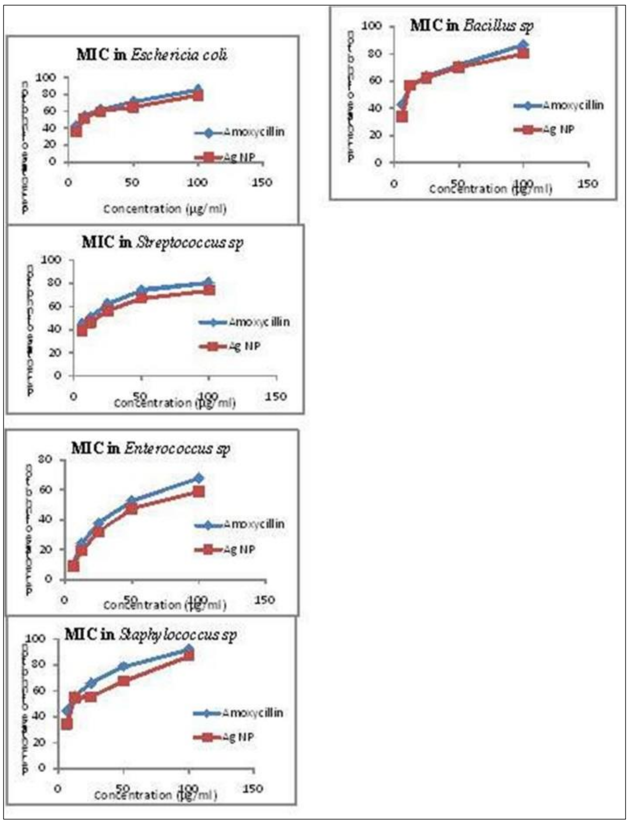

Fig 11 Minimum inhibitory concentration of biosynthesized silver nanoparticles against wound pathogens

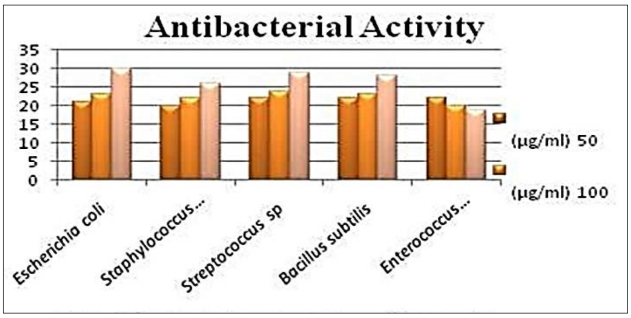

Fig 12 Antibacterial activity of synthesized silver nanoparticles against wound pathogens

Table 4 The minimum inhibitory concentration (MIC) of synthesized silver nanoparticles and amoxicillin antibiotic against wound pathogens

Concentration (µg/ml)	Percentage of inhibition									
	Escherichia coli		*Streptococcus*		*Staphylococcus*		*Bacillus*		*Enterococcus*	
	Std	AgNPs	Std	AgNPs	Std	AgNPs	Std	AgNPs	Std	AgNPs
6.25	42.84	36.11	44.89	39.45	44.5	34.5	42.79	33.84	10.11	8.86
12.5	54.88	51.8	50.89	46.41	56.23	54.5	56.99	56.82	24.33	19.28
25	62.25	59.66	62.24	56.26	65.89	55.42	63.74	62.25	37.41	31.76
50	71.37	65.2	74.22	67.14	78.97	67.59	71.58	69.83	52.78	47.23
100	85.26	78.48	80.55	73.97	92.42	87.18	86.69	80.27	67.94	59.05
MIC(µg/ml)	5.61	16.82	4.28	19.55	1.14	18.22	3.26	11.42	59.14	72.14

CONCLUSION

We have reported the synthesis of silver nanoparticles by *Martynia annua* extracts, which provide simple and efficient ways for the synthesis of nanomaterials. Silver nanoparticles prepared in this process are quite fast and of low cost. The characterization of Ag+ ions exposed to these plant extracts by UV-vis, FT-IR and XRD techniques confirm the reduction of silver ions to silver nanoparticles. The reduced silver ions exhibited efficient antimicrobial and antioxidant activity. Biological synthesized silver nanoparticles could be of immense use in the medical field for their efficient functions.

Conflict of interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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I acknowledge my sincere gratitude to my guide, staffs and my college.

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