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RESEARCH ARTICLE

Synergistic Activity of Green Silver Nanoparticles with Antibiotics

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ABSTRACT

Objective(s): The present work represents the green synthesis of silver nanoparticles using Withania coagulans extract and its antibacterial property. The synergy, additive, bacteriostatic and bactericidal effect of silver nanoparticles was determined against Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Salmonella typhi, and Vibrio cholerae.

Methods: The green silver nanoparticles were characterized by X-ray diffractometry, Transmission Electron Microscopy, Scanning Electron Microscopy and Fourier Transform Infra Red spectroscopy. The Agar dilution, Minimum Inhibitory Concentration and Bacterial Growth Inhibition methods were used for the determination of the antibacterial activity of silver nanoparticles. The Fractional Inhibitory Concentration Index method was performed to check the synergistic activity of conjugated silver nanoparticles.

Results: The Withania coagulans extract were reduced the silver nitrate into silver nanoparticles which was confirmed by color changes and spectral analysis. The silver nanoparticles were crystalline, elemental and spherical. The antibacterial activity was reported in silver nanoparticles which confirmed by zone of inhibition and pores on the surface of bacteria. The conjugated silver nanoparticles with Levofloxacin have synergy and additive behavior against the tested bacteria. Furthermore, bacteriostatic and bactericidal nature of silver nanoparticles was reported in lower (<20 µg/ml) and higher concentration (>50 µg/ml) respectively.

Conclusions: The phenolic compounds of W. coagulans was responsible for the formation of silver nanoparticles. The bacteriostatic and bacteriocidal activity of silver nanoparticles depends upon its concentration. The conjugation of silver nanoparticles with antibiotics may be beneficial due to its synergy and additive effect against the bacteria.

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INTRODUCTION

Nanotechnology is a fascinating area of science which is generating new applications in biotechnology & nano-medicine that encouraging for the development of new kind of nanoparticles [1]. The physical, biological and chemical methods are used for the synthesis of nanoparticles, but chemical and physical methods are not preferred due to high cost, low yield and toxic reducing agents [2-3]. In biological methods microorganism and plants are used for the formation of nanoparticles. The syntheses of nanoparticles by the help of microorganism are not preferred due to release of toxic compounds [4-5]. The plant-based synthesis has gained attention due to its cost-effectiveness, easily availability, easy handling, eco-friendly, medicinal properties and no need for an aseptic environment [6]. The various reports have confirmed that plant extracts synthesized Au, Ag, Pd, and Pt nanoparticles [7]. The AgNPs has been used as antifungal, antibacterial, antiviral, antifouling, antiparasitic, anticancerous agents [8-

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10]. Due to these immense applications AgNPs play major role in the field of nano-medicine. Various reports proved that plants extract have been used for the synthesis of silver nanoparticles. The plant extract of *Lantana camara* [11], *Orange* peel [12], *Chrysanthemum morifolium Ramat* [13], Onion [14], Banana, Neem and *O. tenuiflorum* [15], Coffee and Tea [16] Garlic [17] and *Withania somnifera* [18] have been used for the synthesis of silver nanoparticles. The natural compound such as Kefiran has antibacterial activity [19]. *Withania coagulans* Dunal is a well-known medicinal plant in Indian Ayurveda, belongs to the family Solanaceae. It is found in India, Pakistan, Iran, and Afghanistan. The *Withania coagulans* extract has been used in the diabetes mellitus, blood purification, wound healing, antimicrobial, antifungal, hepatoprotective, hypoglycemic, hypolipidemic, cardiovascular and free radical scavenger [20-21]. The various kinds of literatures have been proved that conjugation of silver nanoparticles with antibiotics enhances antibacterial activity [22-25]. The Levofloxacin is a broad-spectrum antibiotic that are highly effective against bacteria (gram-positive and gram-negative). This antibiotic contains quinolone ring structure (6-fluoro substituent and 7-piperazinyl substituent) and hydrophilic nature. This properties are reduces their tendency to pass through the bacterial cell wall and also inhibits DNA gyrase and bacterial topoisomerase IV. Further Levofloxacin has been used against urinary tract infections (UTI), pyelonephritis, acute bacterial sinusitis and community-acquired pneumonia [26]. Thus, our present work developed a greener method for the formation of silver nanoparticles using *Withania coagulans* and its antibacterial activity. Moreover, the synergistic and additive behavior of conjugated silver nanoparticles with antibiotics (Levofloxacin) was determined against the bacteria.

METHODS

Preparation of Withania coagulans fruits extract

W. coagulans plant was collected from botanical garden of Banaras Hindu University, Varanasi India and authenticated by Prof NK Dubey, Department of Botany, Banaras Hindu University. The specimen voucher no of *W. coagulans* plant is Solana.2020/1. Then plants were dried at 40-45 ^{0}C for 7 days in oven. This fruit was powdered using the grinder and prepared 6 % extract by boiling powder in 500 ml Erlenmeyer flasks containing 100 ml distilled water for 5 minutes. Further extract was filtered using Whatman filter paper (No 1) and filtrates were stored at 4 ^{0}C for further study [30].

Phytochemical Screening

The qualitative tests of phytocompounds (Phenols, Tannins, Phlobatannins, Steroids, Terpenoids, Proteins, Carbohydrates and Saponin) of the extract were analyzed using standard methods [20][21][30].

Determination of Phenols and Tannins

2 ml $FeCl_3$ (2%) was mixed with 1 ml extract (6%) and observe blue-green or black color of the solution. The appearance of blue-green or black color confirmed the presence of phenols and tannin [30].

Determination of Phlobatannins

1 ml extract was mixed with few drops of hydrochloric acids (1%) and boiled for a few minutes. If the solution contains the red precipitate, the extract contains the phlobatannins [30].

Determination of Steroids

1 ml plant extract was mixed with 2 ml chloroform, 2 ml glacial acetic acid and 2 ml Conc $H_2 SO_4$ solution. The presence of greenish color of the solution is indicated the presence of steroids [30].

Determination of Terpenoids

2 ml plant extract, 2 ml chloroform and 1.5 ml Conc. H_2SO_4 was mixed. The development of reddish-brown color indicated the presence of terpenoids in the extract [30].

Determination of Proteins

1 ml extract was mixed with 2 ml ninhydrin (0.2%) solution and boiled for a few minutes. The appearance of violet color indicated the presence of proteins in the extract [20].

Determination of Carbohydrates

1 ml extract was mixed with 2ml Molish reagent and vortex properly. Then added 2 ml H_2SO_4 solution and observed the presence of violet color ring in the solution, this ring confirmed the presence of carbohydrate in the extract [20].

Determination of Saponins

1 ml extract was mixed with 5ml deionized water and shaken vigorously. Then formation of

stable foam confirmed the presence of saponins in the extract [21].

Green synthesis of Silver nanoparticles

6% extract (10ml) was mixed with 1mM $AgNO_3$ (90ml) in 200 ml flask and allowed the reaction at room temperature. This extract reduced the silvernitrate into silver nanoparticles (AgNPs) that was confirmed by visible color changes (yellow to dark brown) of the solution. Then 2 ml of this solution was taken and absorbance was recorded at 200-800nm using UV–Visible spectrophotometer (Systronics, AU-2701). Then solution was centrifuged at 5,000 rpm for 15 minutes and pellets were collected. Further this pellet was washed three times using 5 ml deionized water and centrifuged at 5,000 rpm [28].

Analysis of X- Ray diffraction

The powder XRD was performed for the analysis of crystalline or amorphous nature of silver nanoparticles (AgNPs). The powder XRD model no Bruker Advanced D8, Eco was performed using CuKα radiation (λ = 1.5418 Å) at 2θ angle. Then AgNPs was placed in the sample holder and scanned at a rate of 1° per minute ranges from 30° to 70° [28].

Analysis of Transmission Electron Microscopy

The TEM was performed to check the morphology of AgNPs. The TEM model no. JEOL JEM 200 CX was used in which a drop of AgNPs was placed on grid (carbon coated). This grid was dried under a lamp for overnight. Then transmitted electrons interact with the AgNPs and formed the image which was detected by detector [28].

Analysis of Scanning Electron Microscopy

The SEM was performed for the determination of surface morphology of AgNPs. The SEM model no. JEOL-MODEL 6390 was used for the determination of AgNPs size. Then grid was prepared using silicon wafer and placed small amount of sample powder on the grid and dried under the lamp for overnight. For this analysis accelerating voltage (5-10 KeV) was used. The SEM was also performed for the analysis of effect of AgNPs against bacteria. Then an S. Typhi bacterium was treated with AgNPs for 2 hours [28].

Analysis of Fourier Transforms Infrared Spectroscopy

The FTIR was carried out to check the possible phytocompounds of extract which are responsible for the reduction, capping and stabilization of the AgNPs. The FTIR (Varian Excalibur 3000, Palo Alto, CA) were performed in the range of 4000-500 cm^{-1} to check the possible composition and functional groups that present on the surface of AgNPs [28].

Analysis of Antibacterial Assay

Screening of Antimicrobial Activity

The antibacterial activity of extract, $AgNO_3$ and AgNPs were screened against *Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Salmonella Typhi,* and *Vibrio cholerae* bacteria. The pure bacterial cultures were sub-cultured on Luria broth (agar solidified) medium. Then each bacterial strain was uniformly swabbed on the agar plates. Then 10 µL samples (extract/ $AgNO_3$/AgNPs) were dropped on the agar plates of bacteria. The deionized water was used as control. Then petri-plates were kept at 37 °C for 24 hours. After the incubation inhibition zone around the extract, $AgNO_3$ and AgNPs were measured [29].

Determination of Minimum Inhibitory Concentration

Broth micro-dilution method was performed to check the MIC value of antibiotic Levofloxacin (P) AgNPs (Q) and its combination (P+Q). The McFarland's (0.5) standard bacterial suspension was pipetted into a 96 well microtiter plate. Then variable concentration of sample (P/Q/P+Q) (0, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 µg/ml) were used to check the antimicrobial activity against bacteria *E. coli, P. vulgaris, S. aureus* , S. Typhi, *V. cholerae, and E. faecalis* (concentration 10^6 CFU/ml). Then bacteria were incubated at 37 °C for 24 hours [28-29].

Analysis of Fractional Inhibitory Concentration Index

The Fractional Inhibitory Concentration (FIC) is the ratio of drugs in combination and alone. FIC value of each drug (Levofloxacin and AgNPs) was calculated as follows:

$$\text{FIC of drug P}\left(\text{Levofloxacin}\right) = \left(\text{MIC of drug P in combination}\right) / \left(\text{MIC of Drug P alone}\right)$$

$$\text{FIC of drug Q}\left(\text{AgNPs}\right) = \left(\text{MIC of drug Q in combination}\right) / \left(\text{MIC of drug Q alone}\right)$$

The Fractional Inhibitory Concentration Index (FICI) was calculated as the sum of each

Table 1. The table 1 represents the presence of phytocompounds in the extract.

Plants	Qualitative phytochemical analysis						
	Phenols and Tannins	Carbohydrates	Phlobatannins	Saponins	Steroids	Terpenoids	Proteins
Withania coagulans	+	+	+	-	+	+	+

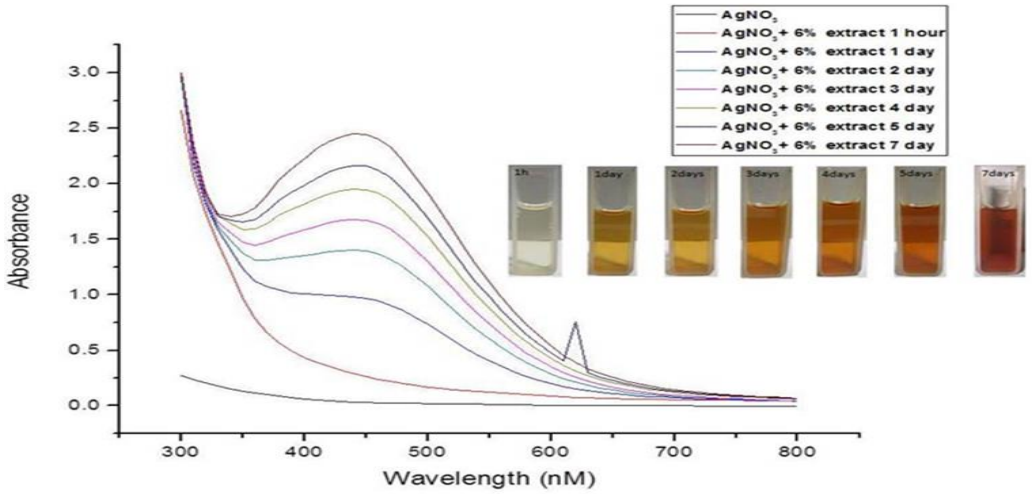

Fig. 1. The images represent the synthesis of silver nanoparticles

FIC of drugs (P&Q). The different values of have determined the behavior of AgNPs when combined with Levofloxacin. If FICI < 0.5, synergy; 0.5 ≤ FICI < 2, additive; and FICI ≥ 2, antagonistic [25].

Analysis of Bacterial Growth Inhibition

The LB media was used for the determination of effect of silver nanoparticles on the bacterial growth. Then S. Typhi bacterial colony was added in LB (10 ml) media and placed at 37 °C, 250 rpm for 24 hours. Then diluted in LB media to maintain the 0.05 $OD_{(600\ nm)}$ (0.1 $OD_{(600)}$ represents the 10^8 cells per ml). The 50 µl AgNPs (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml) solution was mixed with 50 µl LB medium and 0.5 µl S. Typhi bacteria in a 96 microtiter plate. Then absorbance was recorded at 600 nm using Thermo scientific, multiskan Ex, serial Rs. 232 C Elisa reader in different time interval (0, 1, 2, 3, 4, 5, 6 hours) [27].

RESULTS

Phytochemical Screening

The phytochemical screening results confirmed that *W. coagulans* extract contains various phytocompounds such as phenols, tannin, phlobatannins, carbohydrates, proteins, steroids and terpenoids while saponins were absent (Table 1).

Green synthesis of Silver nanoparticles

The plant extracts reduced silvernitrate into silver nanoparticles that was confirmed by visible color changes (light yellow to dark brown). The spectral analysis results confirmed that silver nanoparticles synthesis was started after 1 hour of reaction and synthesis was completed after 7 days of reaction (Fig. 1).

Analysis of X- Ray diffraction

The XRD result of silver nanoparticles was contains planes at 111, 200 and 220. These planes confirmed the formation of crystalline silver nanoparticles which was matched by standard silver planes (Fig. 2).

Analysis of Transmission Electron Microscopy

The TEM results confirmed that silver nanoparticles were spherical shape (Fig. 3A), the diffraction ring confirmed that silver nanoparticles was polycrystalline (Fig. 3B). The variable size of

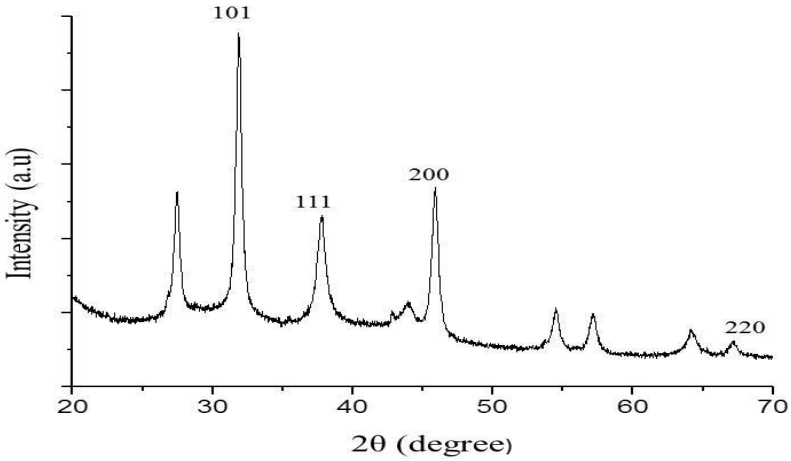

Fig. 2. The image represents the crystalline silver nanoparticles

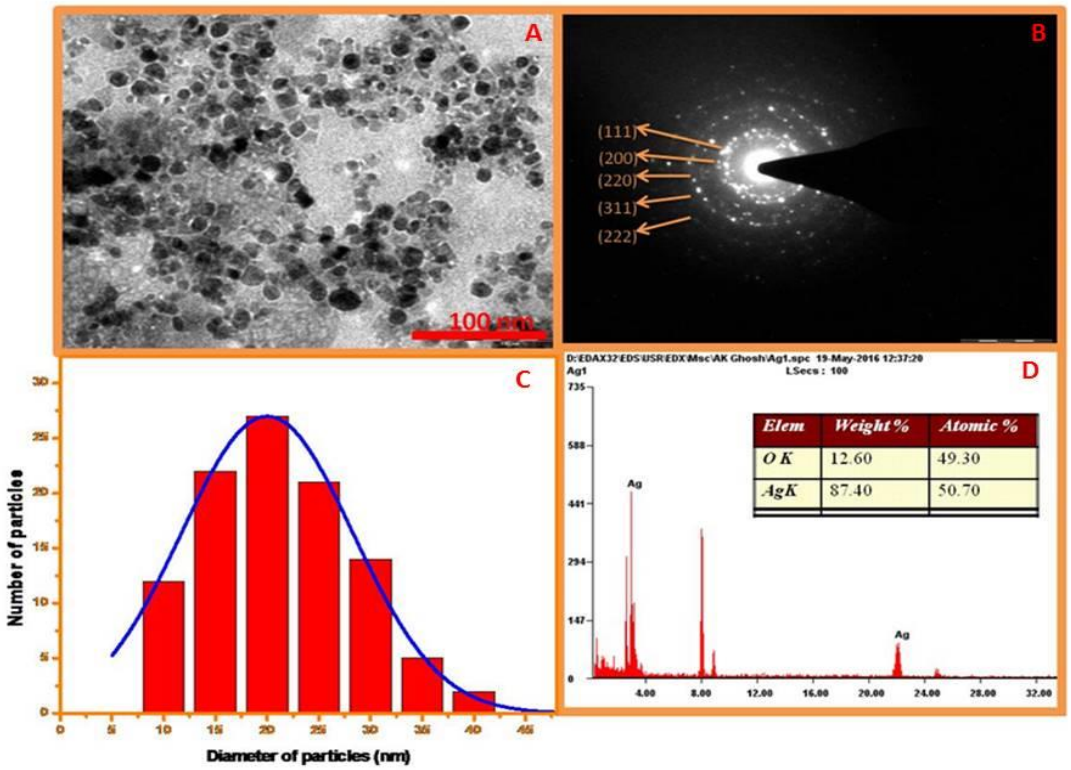

Fig. 3. The images represent the spherical shape (A), fcc crystal of silver (B), variable size (C) and elemental nature of silver nanoparticles (D)

silver nanoparticles was formed that size ranged from 10-40 nm. But 20 nm size particles were maximum in number (Fig. 3C). The appearance of intense peaks at 3 KeV were proved that silver nanoparticles was elemental (Fig. 3D).

Analysis of Scanning Electron Microscopy

The SEM results confirmed that silver nanoparticles were spherical shape (Fig. 4). The antibacterial activity of silver nanoparticles was also confirmed by formation of pores on the surface of S.

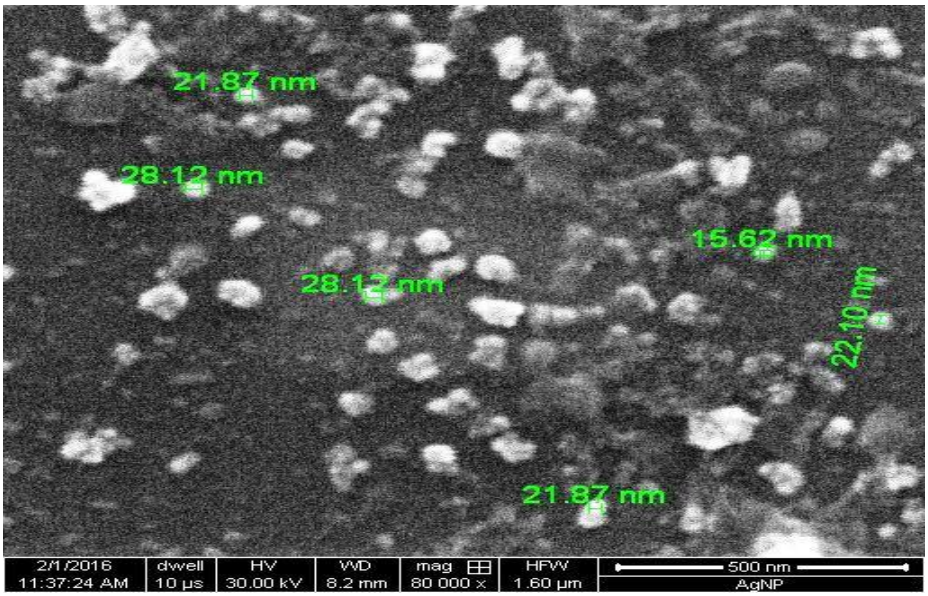

Fig. 4. The image represents the spherical shape of silver nanoparticles

Fig. 5. The image represents the normal S. Typhi (A) and treated S. Typhi with silver nanoparticles (B)

Typhi bacteria. This silver nanoparticle was created the pores after 2 hours of silver nanoparticles treatment [Fig. 5].

Analysis of Fourier Transforms Infrared Spectroscopy

The FTIR result of AgNPs was studied which indicated the absorption peaks location on 3477, 3349, 2917, 2347, 2208, 2003, 1657, 1540 cm^{-1} (Fig. 3). The band at 3477, 3349, 2917, 2347, 2208, 2003 and 1657cm^{-1} were assigned to O–H stretch (phenolic compounds), N-H stretch (primary, secondary amines and amides), C-H stretching (methyl groups), H-C=O: stretching (aldehydes), C≡N stretching (nitriles), C≡C stretching (alkynes), C=O stretching (carbonyl groups) (Fig. 6). The presence of OH groups of the extract was involved in reduction of silvernitrate into silver nanoparticles because polyphenols (phenols and flavonoids) contain sufficient hydroxyl and carboxyl groups to form complex with metals ions. The carbonyl group (CO) of extract have strong binding tendency with silver leads to formation of a layer. This layer prevented agglomeration of silver particles and also act as capping agent that provide stability to silver nanoparticles [31].

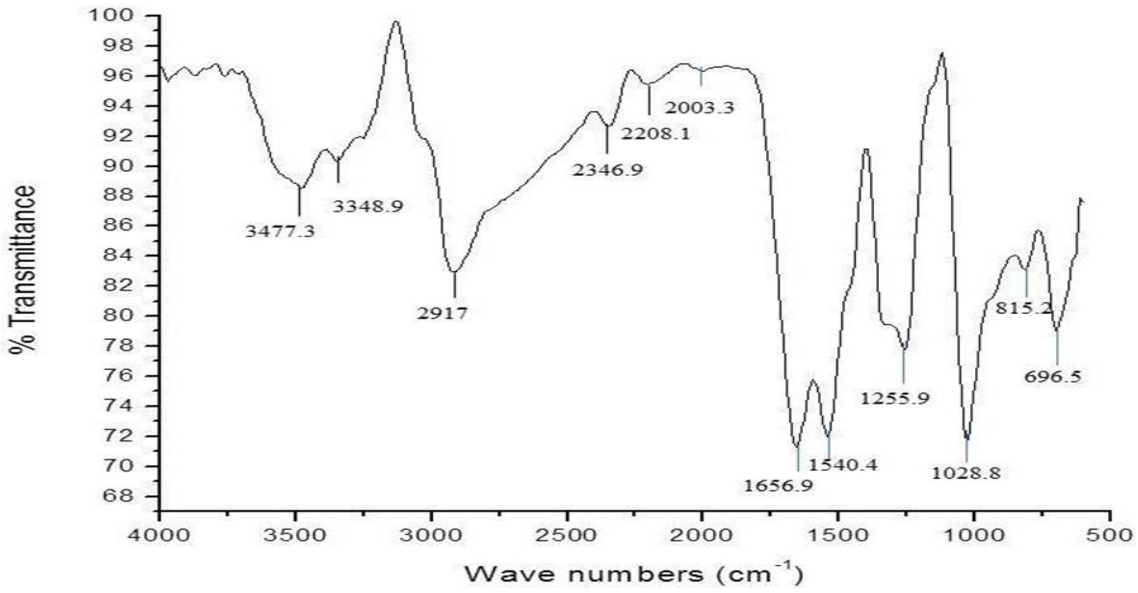

Fig. 6. The image represents the presence of functional groups on the surface of silver nanoparticles

Analysis of Antimicrobial Assay

Screening of Antimicrobial Activity

The results indicated that zone of inhibition of $AgNO_3$ and AgNPs against the bacteria which confirmed the antibacterial activity of $AgNO_3$ and AgNPs. The AgNPs have a zone of inhibition 21 mm *(E.coli),* 43 mm (*Vibrio cholerae*), 21 mm (*E. faecalis*), 14 mm (*S. aureus*), 32 mm (*S. typhi*), 21 mm (*P. vulgaris*). The $AgNO_3$ has inhibition zone 15 mm (*E.coli*), 17 mm (*Vibrio cholerae*), 10 mm (*E. faecalis*), 11 mm (*S. aureus*), 11 mm (S. Typhi), 11 mm (*P. vulgaris*). The AgNPs have strong antibacterial activity in comparison to $AgNO_3$ and revealed a wider bacterial inhibition zone than $AgNO_3$. No antibacterial activity was reported in extract while deionized water was used as control (Fig. 7 and Table 2).

Analysis of Minimum Inhibitory Concentration

The MIC value of Levofloxacin (P) was 2µg/ml (*E.coli*), 8µg/ml (*Vibrio cholerae*), 8µg/ml (*E. faecalis*), 1µg/ml (*S. aureus*), 1µg/ml (S. Typhi), 32µg/ml (*P. vulgaris*). The MIC value of AgNPs (Q) was 16µg/ml (*E.coli*), 16µg/ml (*Vibrio cholerae*), 32µg/ml (*E. faecalis*), 4µg/ml (*S. aureus*), 32µg/ml (S. Typhi), 16µg/ml (*P. vulgaris*). The MIC value of conjugated AgNPs (P&Q) was 0.5µg/ml (*E.coli*), 8µg/ml (*Vibrio cholerae*), 0.25µg/ml (*E. faecalis*), 0.25µg/ml (*S. aureus*), 0.25µg/ml (S. Typhi), 16µg/ml (*P. vulgaris*). This result proved that conjugated silver nanoparticles have greater MIC value than P and Q (Table3).

Analysis of Fractional Inhibitory Concentration Index

The results confirmed that FIC value of Levofloxacin (P) was 0.25 (*E.coli*), 1 (*Vibrio cholerae*), 0.031 (*E. faecalis*), 0.25 (*S. aureus*), 0.25 (S. Typhi), 0.5 (*P. vulgaris*). The FIC value of AgNPs (Q) was 0.031 (*E.coli*), 0.5 (*Vibrio cholerae*), 0.007 (*E. faecalis*), 0.062 (*S. aureus*), 0.007 (S. Typhi), 0.5 (*P. vulgaris*). The FICI value of conjugated silver nanoparticles was 0.281 (*E.coli*), 1.5 (*Vibrio cholerae*), 0.038 (*E. faecalis*), 0.312 (*S. aureus*), 0.257 (S. Typhi), 1 (*P. vulgaris*). This FICI value proved that conjugated silver nanoparticles were represented synergistic activity against *E.coli, E. faecalis, S. aureus* and S. Typhi while additive effect against *V. cholerae* and *P. vulgaris* bacteria (Table 3).

Analysis of Bacterial Growth Inhibition

The result confirmed that AgNPs have antibacterial activity after 30 minutes while the antibacterial properties enhance as time increases (from 2-5 hours) against the S. Typhi bacteria. When the concentration of AgNPs was increased the growth curve of S.typhi bacteria was decreased. The 20µg/ml AgNPs decreases the growth of

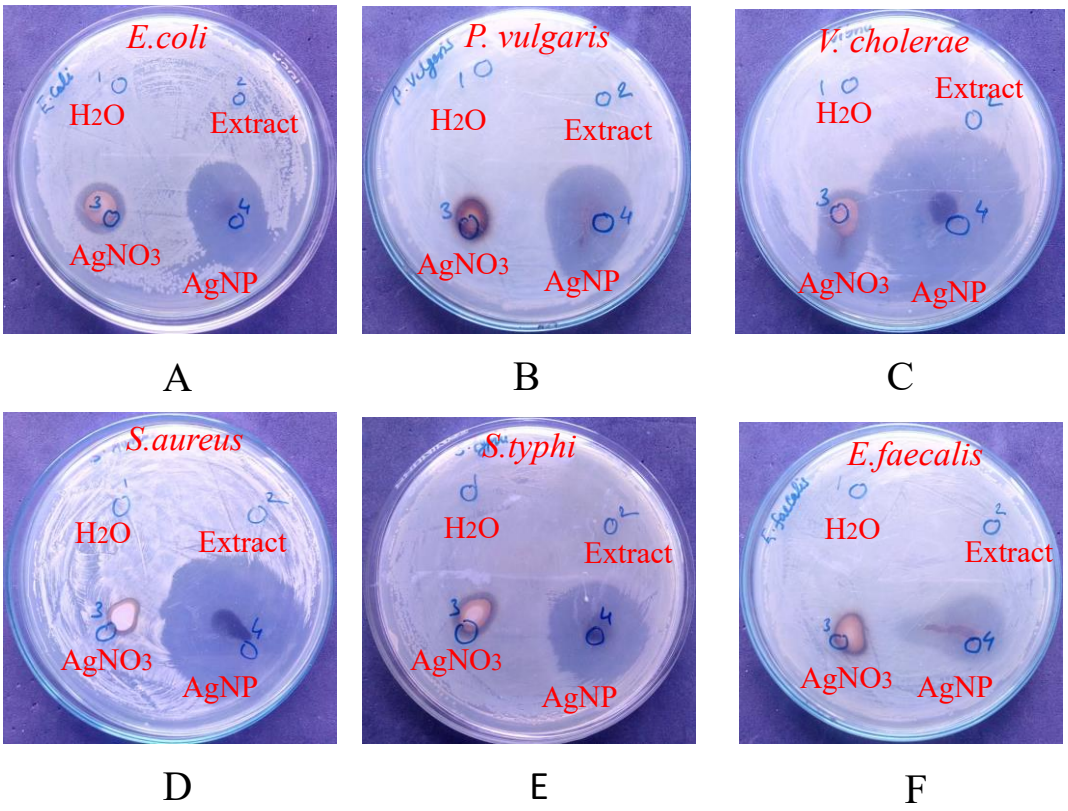

Fig. 7. The image represents the antibacterial activity of silver nanoparticles, silver nitrates and extract against the bacteria

Table 2. The table 2 represents the antibacterial activity of silver nanoparticles, silver nitrates and extract

S.N	Bacteria	*Withania coagulans* extract	Inhibition zone (mm)	
			$AgNO_3$	AgNPs
1	*E.coli*	NA	15	21
2	*Vibrio cholerae*	NA	17	43
3	*E. faecalis*	NA	10	21
4	*S. aureus*	NA	11	14
5	*S. typhi*	NA	11	32
6	*P. vulgaris*	NA	11	21

S.typhi bacteria while above the 50µg/ml AgNPs the number of bacterial colony was killed. These results confirmed silver nanoparticles below the 20µg/ml act as bacteriostatic and above the 50µg/ml act as bactericidal (Fig. 8).

DISCUSSION

The phenolic compounds of *W. coagulans* is reduced the silvernitrate into Silver Nanoparticles (AgNPs) (Fig. 6). The synthesis of AgNPs was confirmed by color changes of solution and UV-visible spectroscopy [Fig. 1]. The AgNPs are crystalline in nature which proved by the SEM [Fig. 2]. The TEM results proved that silver nanoparticles were spherical shape [Fig. 3A], monocrystalline [Fig. 3B], variable size [3C] and elemental [Fig. 3D]. The SEM results also proved that silver nanoparticles are spherical shape [Fig. 4].

Table 3. The table represents the Minimum Inhibitory Concentration, Fractional Inhibitory Concentration and Fractional Inhibitory Concentration Index of Levofloxacin (P), AgNPs (Q), and its combination (P+Q)

S.N	Bacteria	Minimum Inhibitory Concentration (MIC) (µg/ml)			Fractional Inhibitory Concentration (FIC) (µg/ml)		Fractional Inhibitory Concentration Index (FICI) (µg/ml)
		P	Q	P + Q	P	Q	Combination (P, Q)
1	*E.coli*	2	16	0.5	0.25	0.031	0.281 (S)
2	*V. cholerae*	8	16	8	1	0.5	1.5 (A)
3	*E. faecalis*	8	32	0.25	0.031	0.007	0.038 (S)
4	*S. aureus*	1	4	0.25	0.25	0.062	0.312 (S)
5	*S. typhi*	1	32	0.25	0.25	0.007	0.257 (S)
6	*P. vulgaris*	32	16	16	0.5	0.5	1 (A)

Note: Synergy (S), Additive (A)

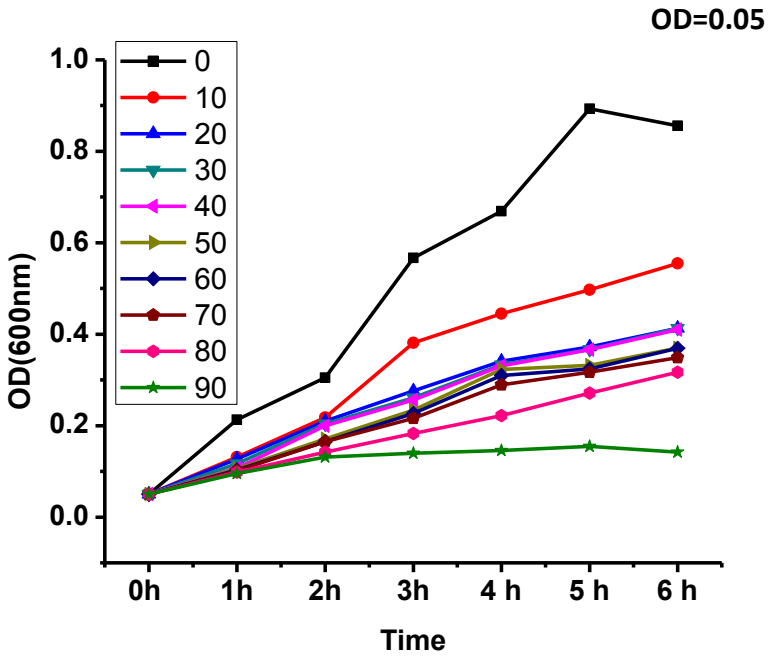

Fig. 8. The image represents the time dependent effect of silver nanoparticles on the growth of S. Typhi bacteria.

The AgNPs have created the pores on the bacterial surface by damaging the cell wall which confirmed by the SEM [Fig. 5]. The FTIR results confirmed that *Withania coagulans* contains different types of phytocompounds such as, primary, secondary amines and amides, aldehydes, alkynes, phenolic compounds, flavonoids and tannins [Fig. 6]. The inhibition zone against S. Typhi, *E.coli, P. vulgaris, S. aureus, V. cholerae*, and *E. faecalis* bacteria were confirmed antibacterial activity of AgNPs [Fig. 7]. Levofloxacin, AgNPs and its conjugation have antibacterial activity which was confirmed by MIC result. As we know lower MIC values higher will be the antibacterial activity. The conjugated

silver nanoparticles with antibiotics (AgNPs-Levo) has lower MIC value than AgNPs and Levofloxacin that confirmed that AgNPs-Levo have greater antibacterial activity [Table 3]. The FICI values of AgNPs-Levo confirmed that this conjugation is beneficial due to its dual behavior such as synergistic and additive when compared with AgNPs and Levofloxacin alone [Table 3]. This enhanced antibacterial activity of AgNPs-Levo due to the hydrophobic nature of AgNPs, the release of Ag+ and mode of action of AgNPs and Levofloxacin against the bacteria [8]. The results of bacterial growth inhibition against the S. Typhi have confirmed that bacteriostatic and bactericidal property of AgNPs depends upon the concentration of AgNPs. The lower concentration of AgNPs (<20 µg/ml) act as bacteriostatic while higher the concentration of AgNPs (>50 µg/ml) act as bactericidal [Fig. 8].

CONCLUSION

The present work describes the simple, fast, cheap and eco-friendly green synthesis of AgNPs using the *Withania coagulans*. The plant extract contains phenols that are responsible for the reduction of $AgNO_3$ into AgNPs. The AgNPs has bacteriostatic effect. The AgNPs-Levo has strong antibacterial activity than AgNPs and Levofloxacin against selected bacteria. The synergy and additive behavior of AgNPs-Levo occurs due to the mode of action of AgNPs and antibiotic. This conjugation is always beneficial because bacteria will not develop resistant against antibiotics.

CONFLICT OF INTEREST

The authors were declared that there is no conflict of interest and this research work is genuine.

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