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RESEARCH ARTICLE

Biogenic Synthesis of Silver Nanoparticles Using *Terminalia chebula* Retz. Leaf Extract and Evaluation of Biological Activities

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Abstract

Nanoparticles have been used in various fields of science and technology ranging from material science to biotechnology. The formation of nanoparticles has been confirmed through UV-visible spectroscopy (at 420 nm) by the change of colour representing surface plasmon resonance. The synthesis of silver nanoparticles by a biogenic method is a novel approach due to its cost-effective, eco-friendly, and large-scale production possibilities. In the present study, silver nanoparticles (TC-AgNPs) were successfully synthesized using *Terminalia chebula* Retz. (*T. chebula*) leaf extract. Characterization of green synthesized silver nanoparticles was performed using UV-visible *spectroscopy*, Fourier transforms infrared (FTIR) spectroscopy, and X-ray diffraction (XRD). The formation of nanoparticles has been confirmed through UV-visible spectroscopy (at 420 nm) by the change of colour representing surface plasmon resonance. The crystalline face-centred cubic property of the biosynthesized silver nanoparticles was established using XRD analysis. The XRD data gave the average particle size of 6.1 nm. The functional groups such as -OH, C=O, =NH were found responsible for reducing silver ions and helping to stabilize nanoparticles which were analysed using FTIR spectroscopy. As the silver nanoparticles possess diverse applications, TC-AgNPs were investigated for antioxidant, antibacterial, and cytotoxic activity. The results showed TC-AgNPs showed potential antioxidant (IC_{50}=312.8 ± 2.28 µg/mL) and antibacterial activities against four pathogenic bacteria like *Staphylococcus aureus*, *Acinetobacter baumannii*, *Salmonella typhi*, and *Escherichia coli*. Also, the silver nanoparticles exhibited moderate cytotoxicity (LC_{50}= 477.53 ± 0.684 µg/mL) against brine shrimps nauplii in a dose-dependent manner.

Keywords:*T. chebula,* silver nanoparticles, brine shrimp toxicity, XRD, FTIR, antibacterial, leaf extract, TC-AgNPs

Introduction

Multidrug resistance is a major health concern in the medical field [1]. Researchers are trying to avoid such problems by developing new antibacterial agents. Nanotechnology gives the best approach for developing novel antibacterial agents or substitutes [2]. Nanotechnology is the most emerging field of research. It deals with the preparation and applications of nanoscopic substances having the particle size 1-100 nm[3]. Being at the transition between bulk materials and the smallest atoms or molecules, nanomaterials show unique properties that don't occur in either case. For instance, silver nanoparticles are better and more efficient antimicrobial agents as compared with the bulk material[4]. Nowadays silver nanoparticles are widely used as an antimicrobial coating on various fibres, metals, plastics, medical utensils, etc. to protect against bacteria and fungi. [5-6] Such unique properties of nanoparticles can be discussed based on their proper size, morphology, crystallinity, shape, and chemical composition [7].

Nanoparticles may be metallic (Nanorod or nanosphere of Au and Ag), semiconducting (InP, GaN, CdSe, Si quantum dots), insulating (SiO_2 nanosphere), and hybrid type (Au core encapsulated with SiO_2 shell). [8]Among various nanoparticles, silver nanoparticles are in great application because of their chemical stability, high conductivity, catalytic activities, and localized surface plasmon resonance [9].They are also important components of environmental pollution [10] and the key ingredients of industrial products like paints, ceramics, plastics, magnetic articles, etc. Green synthesized AgNPs have diverse applications in the field of antibacterial, antiviral, antifungal, anti-inflammatory, antitumor, cosmetics, etc [11]. Biogenic synthesis of AgNPs is superior to Physico-chemical methods due to capital extensive with many defects such as using of toxic solvents, production of hazardous byproducts and the imperfection of the surface structure [44]. The chemical method of synthesis involved the application of diverse chemicals that may increase the particle reactivity and toxicity which might harm the human health and the environment due to the composition in more possible ways and lack of predictability [44].

Silver nanoparticles are obtained from both the top-down and bottom-up approaches [12].There are three major methods for the synthesis of silver nanoparticles like physical, chemical, and biological methods. Many physical and chemical methods for the production of silver nanoparticles (AgNPs) involve the use of large amounts of toxic substances and are also expensive. They may have various environmental and biological hazards [10]. Therefore, the synthesis of silver nanoparticles from biological methods is becoming more reliable and the appropriate method due to their biocompatibility. The different bioactive constituents present in the living system help in the direct reduction of silver ions and also act as a good natural capping agent. Mostly the bio-constituents present in the biological source like phenols, flavonoids, terpenoids, quinines tannins, etc; are playing a vital role in those activities [13-14].

While studying several plants with their medicinal values, the plant *T. chebula* Retz. has been reported as a potent plant against several simple to life-threatening diseases [15]. The plant contains a large variety of phytoconstituents such as polyphenols, terpenes, anthocyanins, flavonoids, alkaloids, and glycosides, to show the high number of medicinal values [16]. A group of researchers have reported numeroushydrolysable tannins (gallic acid, chebulagic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulinic acid, 1,2,3,4,6-penta-O-galloyl-β-D-glucose, 1,6-di-O-galloyl-D-glucose, casuarinin, 3,4,6-tri-o-glloyl-D-glucose, terchebulin) from *T. chebula* [17]. The present study aims the synthesis offine crystalline silver nanoparticles using *T. chebula* leaf extract collected from Surkhet district of Nepal. In this method, the bio-constituents present in the plant play a vital role in both reducing and capping agents during nanoparticle synthesis. And this method is considered superior and eco-friendly to other Physico-chemical methods because it is conducted without the direct involvement of hazardous chemicals. In addition to this, the study also evaluates the different biological activities like antibacterial, antioxidant and lethality activity of thus prepared silver nanoparticles and plant extract.

Materials and methods

Chemicals and materials

The leaves of *T. chebula* were collected from the Surkhet district and identified at National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal. Laboratory grade chemicals such as DPPH (2, 2-diphenyl-1-picrylhydrazyl), Folin-Ciocalteu reagent (FCR), silver nitrate ($AgNO_3$), Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), and other chemicals were used throughout the experiment.

Preparation of extract

500 g leaves of *T. chebula*were first dried under dry shade at least for 2 weeks. The dried leaves were ground into powdered form with the help of a grinding machine. The methanolic extract was prepared by cold percolation in which 200 g of leaf powder was dipped into 600 mL of pure methanol for 72 h. After filtration using Whatman No.1 filter paper, the solvent was evaporated by a rotary evaporator at 40 ºC. The

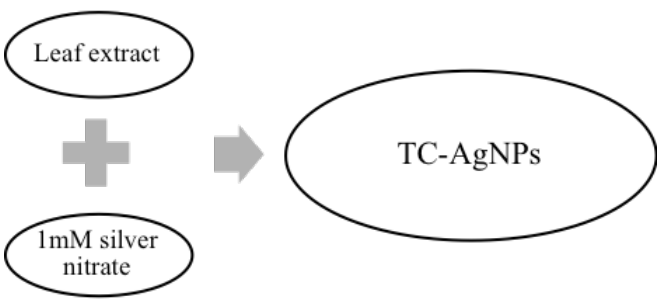
dried extract was stored in the refrigerator at 4ºC until needed [18].

Qualitative phytochemical analysis

The extract obtained was subjected to qualitativechemical tests to detect the presence of variouschemical constituents. Major secondary metabolites classessuch as alkaloids, flavonoids, phenol, coumarins, terpenoids, tannins, saponins, steroids, glycosides, vitamin-C, etc. were screened [19].

Biogenic synthesis of silver nanoparticles

The aqueous leaf extract was prepared by warming 3 g of powdered sample in 100 mL of deionized water for 20 min at 80 ºC. Then after it was hot filtered with the help of Whatman No. 1 filter paper [20]. The appropriate volume of plant extract can reduce the silver ion (Ag^+) at room temperature. In the first set of analyses, various volumes of the plant extract were allowed to react with the 1 mM silver nitrate solution in the ratio of 1:9, 2:8, 3:7, 4:6, and 5:5 at room temperature. The solution was kept inthe dark after 20 minutes agitation was done with the help of a magnetic stirrer. The formation of silver nanoparticles was preliminarily confirmed by the colour change from light yellow to reddish-brown. The UV-spectra were taken at three intervals of time i.e., at 0 h, 4 h, and 24 h in the UV-visible range of 300-700 nm. The optimized concentration of plant extract against 1 mM silver nitrate solution and the best nanoparticle formation time was estimated at a 1:9 ratio with a reaction time of 24 h.



In the second set of analyses, 10 mL of plant extract was mixed with 90 mL of 1 mM silver nitrate solution and it was agitated properly using a magnetic stirrer for 20 minutes. The formation of the silver nanoparticles was preliminarily confirmed by the colour change from light yellow to a reddish-brown solution. After that, it was kept properly in dark for 24 h. Finally, the UV visible spectroscopy was taken under the range 300 nm-700 nm. The method of separation or collection is very important as the variability in dispersive ability and morphology of the nanoparticles rely on the way it proceeds. The nanoparticles were purified using the ethanol precipitation method [21].

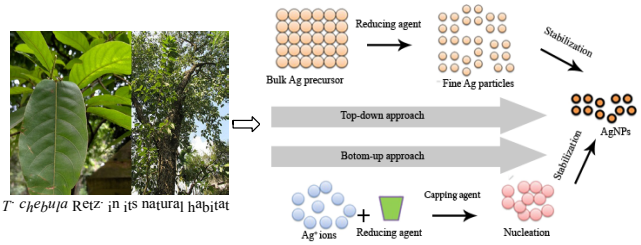


Figure 1:*Schematic representation of AgNPs synthesis through bottom-up and top-down approaches* [12].

Characterization of TC-AgNPs

UV-Vis Spectroscopy

The formation of the silver nanoparticles was confirmed by analyzing the absorption band. The reduction of silver ion (Ag+) was monitored with the help of the SPECORD 200 PLUS, Analytic Jena (An Endress+Hauser Company) UV/VIS Spectrophotometer scanning in the range of 300-700 nm at a medium scanning rate and resolution of 1 nm. Deionized water was used as the reference for the baseline correction. The sample was loaded into a 2 mL quartz cuvette with a path length of 1 cm.

Fourier transform infrared spectroscopy (FTIR)

The organic functional groups present in the sample of plant extract and silver nanoparticles were analyzed using Shimadzu IRTracer-100 from 4000 to 400 cm^{-1}. It helped to compare the IR spectra of missing functional groups of plant extract during the green synthesis of silver nanoparticles.

X-ray diffraction (XRD)

X-ray diffractogram was obtained usingthe Rigaku D/MAX-2500/pc diffractometer with monochromator Cu Kα radiation of wavelength 1.54060 Å with 30 mA current and 40 kV voltage in a scan rate of 10°/minute across the 2θ angle ranging from 0 to 90. This diffractogram was used to confirm the crystal nature of silver nanoparticles and predict their mean size.

Biological activities

Antibacterial activity

The antibacterial activity of the plant extract was performed by the agar well diffusion method [22]. And for the antimicrobial activity of green synthesized silver nanoparticles, the disk diffusion method was followed [23]. And the corresponding value of the zone of inhibition was evaluated. The antibacterial activity of the plant extract and the silver nanoparticles were tested against bacteria *Staphylococcus aureus*(ATCC 25923), *Escherichia coli* (ATCC 3292), *Acinetobacter baumannii* (ATCC 19606), *Klebsiella pneumoniae* (ATCC 700603), and *Salmonella typhii* (ATCC 14028) and *Shigella sonnei* (ATCC 25931). The direct colony suspension method was used to prepare the inoculum of each test organism. The small pinch of the bacterial colony was transferred to 2 mL of Muller Hinton broth solution and was left in an incubator for 18-20 h for its growth. The suspension was homogenized using a vortex shaker. The turbidity of the resulting suspension was adjusted to achieve turbidity equivalent to that of 0.5 McFarland Standard.

Antibacterial activity of plant extract

A sterile cotton swab was used to evenly distribute bacterial culture drawn from respective inoculums over the Petri plate containing MHA. Four wells of 8 mm diameter were then made in the inoculated plates using a sterile cork borer. Then, 100 µL of each plant sample (of 50 mg/mL in 50 % DMSO) was introduced into the well with 50 % DMSO as negative control and 1 mg/mLof neomycin antibiotic as a positive control. The plates were left in upright condition with lids closed for half an hour so that the test solutions were diffused into the media. The inoculated plates were then incubated at 37 ºC for 24 h. Finally, the zone of inhibition was measured using a scale ruler.

Antibacterial activity of silver nanoparticles

Powdered TC-AgNPs was dissolved at a concentration of 50 mg/mL 5 % DMSO and sonicated for 30 minutes at 25ºC to prepare a stock solution. Paper discs (1 mg TC-AgNPs/disc) were prepared by adding 20 µL of TC-AgNPs stock solution to filter paper discs (6 mm) and dried for 15 min. Furthermore, a different disc of plant extract, 5 % DMSO as negative control and 1 mg/mL neomycin as a positive control was also prepared and collected in different vials and stored at 4 ºC. To perform the antibacterial activity of TC-AgNPs, a sterile cotton swab was used to evenly distribute bacterial culture drawn from respective inoculums over the Petri plate containing MHA. Then paper discs for TC-AgNPs, negative control, positive control, and plant extract were placed on the surface of plates and incubated for 24 h at 37 ºC. Finally, the zone of inhibition was measured using a scale ruler.

Brine shrimp lethality bioassay

The brine shrimp toxicity assay was performed by the standard protocol described by Meyer et al. (1982) [24].

Quantitative analysis

The plant extract was analyzed quantitatively to determine the total phenolic [25],total flavonoid [26], and anti-oxidant activity [27] in comparison to different standard reference compounds. To determine the total phenolic content and flavonoid content, gallic acid and quercetin were used as a standard for the construction of the calibration curve as shown in figure 2.

Antioxidant activity (DPPH assay)

Antioxidant activity was determined using a DPPH assay. It is one of the best and most well-known and easy methods for detecting antioxidant activity. Here, the DPPH was scavenged by the hydrogen atom present in the plant sample. DPPH radical assay was performed for each sample by using quercetin as a standard. The linear regression for the radical scavenging versus concentration was evaluated for determining 50% inhibition of DPPH activity (IC_{50}) values for each sample. The higher value of IC_{50} indicates lower antioxidant activity and also lower IC_{50} value indicates higher antioxidant activity. The corresponding IC_{50} values of the samples were determined using GraphPad Prism version 8.0.1 software.

Furthermore, the aqueous extract of *T. chebula* was used to synthesize silver nanoparticles. The previous study has reported that the plant extract contains large amounts of secondary metabolites which are

responsible for the metal ion reduction and shows the best capping activity. Hence, the antioxidant property of the silver nanoparticles was evaluated. A range of different concentrations of nanoparticles was prepared by dissolving in 5 % DMSO the plot of percent radical scavenging versus concentration of the plant extract and silver nanoparticle is shown in figure 7.

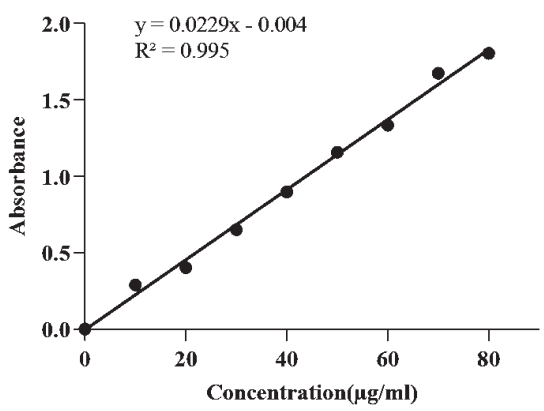
Results and discussion

Qualitative phytochemical analysis

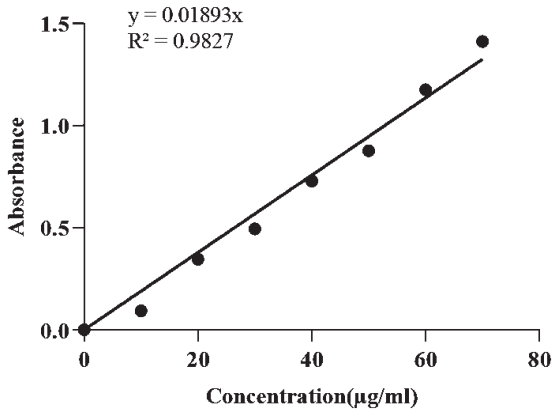
The phytochemical analysis was performed according to the procedure given by Whankar et al. (2015) [19]. The methanolic extract of the *T. chebula*showed the presence of alkaloids, flavonoids, phenolics, tannins, saponins, glycosides, and carbohydrates. Other metabolites like terpenoids, steroids, and carbohydrates were absent. The above results closely approached the literature review of the plants [28-29]. The presence or absence of secondary metabolites in plants also depends on the collection time, altitude, temperature, geographical location, the solvent used environmental conditions, extraction procedures, etc [30].

Determination of total phenolic content (TPC) and total flavonoid content (TFC)in plant extract

TPC was determined using the Folin-Ciocalteu method and expressed in terms of gallic acid equivalent whereas TFC was determined using aluminium chloride colorimetric assay. TFC value was expressed in terms of quercetin equivalenttoa plant extract and calculated with the help of the standard calibration curve shown in figure 2.



a) Calibration curve for gallic acid



b) Calibration curve for quercetin

Figure 2: *Standard calibration curves for gallic acid (a) and quercetin (b)*

TPC value for *T. chebula* was found to be 149.22 ±1.85 mg GAE/g. Arya et al. (2012) have reported that the total phenolic content present in the methanolic extract of *T. chebula* was 266.16 ± 7.81 mg GAE/g [31]. The value of TPC was somehow different compared to the reported in the literature.The plants having a higher value of total phenolic content has gained the major interest of world-class natural product researcher due to their major antioxidant property. Antioxidant metabolites present in the plants help the prevention of chronic and oxidative stress-related disorders such as cancers, cardiovascular and neurodegenerative diseases [32]. Plant extract rich in total phenolic content also impart high antioxidant activity.

The higher value of total flavonoid content present in the plants is considered a high antioxidant activity. The flavonoids rich natural sources are found active against antibacterial, antiviral, and anti-inflammatory as they are regarded as free radical scavengers. Such plants with high flavonoid content can behave as a potential reducing agent and protect from oxidative damage. And also, they are very important chemical reducers and capping agents in the green synthesis of silver nanoparticles [33].

Biogenic synthesis of silver nanoparticles from TC extract

Silver nanoparticles (AgNPs) were synthesized from an aqueous extract of *T. chebula* leaves. The preliminary confirmation for the formation of

nanoparticles was by the colour change from light yellow to reddish-brown. After that, it was kept properly in dark for 24 h. Finally, the UV visible spectroscopy was taken under the range of 300 to 700 nm. The nanoparticles were purified using the ethanol precipitation method.

Figure 3: *Visible observation of colour change of plant extract after addition of* $AgNO_3$

The visible colour change of the nanoparticle solution also best matched with Espenti et al. (2016) which preliminary confirmed the formation of silver nanoparticles [15]. The presence of visible colour change from yellowish extract solution to reddish-brown colour can be depicted based on the local surface Plasmon resonance (LSPR). When the oscillation frequency of the electric field vector matches with the electronic oscillation and maximum absorbance takes place and LSPR occurs [34].

Characterization of silver nanoparticles

UV-visible spectroscopy

After visual confirmation by detecting a colour change in the biosynthesis of TC-AgNPs, the samples were exposed to spectral analysis. The bio-reduction of the Ag^+ions in the aqueous extract was monitored with UV-visible spectra.The reaction kinetics was observed taking different time intervals for nanoparticle syntheses like 0 h, 4 h, and 24 h. The UV-visible spectra of the reaction mixture were recorded for 24 h at varied time intervals. After 24 h, there was no further requirement for monitoring reaction kinetics as the nanoparticles synthesized werestable with a similar UV spectrum. The highest absorbance peak was detected at 420 nm at 24 h shown in Figure .

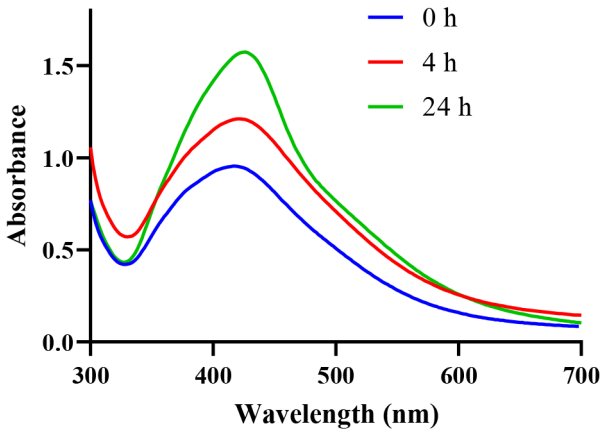


Figure 4: *UV-visible spectral analysis of the biosynthesized silver nanoparticles*

After the green synthesis of silver nanoparticles, the reaction kinetics was initially monitored by UV-visible spectroscopy in the wavelength range of 300-700 nm. Usually, due to the excitation of the free electrons, nanoparticles would show surface plasmon resonance. The most favourable time duration for the formation of stable silver nanoparticles was 24 h in preference to 0 h and 4 h.

The change in the pH of the reaction mixture can change the morphology, size surface area, and property of the nanoparticles. So, the preparation of the nanoparticles was also monitored under varied basic pHadjusted by dropwise addition of hydrochloric acid (HCl) and sodium hydroxide (NaOH).

The maximum and sharp absorbance peak was found at the pH of 10 as compared with other basic pH values. The result is very satisfying to the study by Edison et al. (2012) [35]. The redshift in a basic medium indicates a relatively large particle size compared with in case of the neutral or acidic medium.

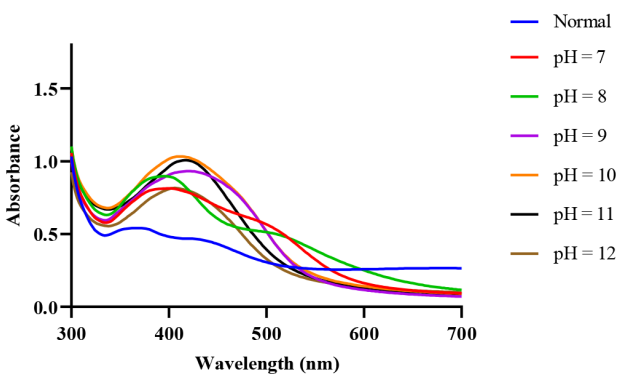


Figure 5: *Synthesis of AgNPs based on pH change*

The intensity of the SPR peaks tends to increase when the pH value increases. The broad center peak assigned at 420 nm indicates the slow reduction of silver ions to silver nanoparticles. These results can be explained based on the effects of pHon the agglomeration, isolation, interfacial free energy, the net charge of the complexing agent, dissociation, etc. [36,37].

Fourier-transform infrared (FTIR) spectroscopy

Further, TC extract and TC-AgNPs were subjected to FTIR analysis, and the results were presented in figure 6.FTIR spectra are very much helpful for identifying the functional group present in the sample. As compared with the absorption peaks of TC extract, spectra of TC-AgNPs showed fewer peaks. The absorption peaks were found for -OH, -CH stretching, -NH bending, N-O stretching, and C-OH stretching at 3254, 2930, 1624, 1525, and 1020 cm^{-1} in the case of TC-AgNPs and for -OH, -CH stretching, symmetrical C=O, C=O stretching, stretching C=C, bending -OH, C-O stretching and C-OH stretching at 3277, 2930, 2325, 1713, 1599, 1328, 1199, and 1018 cm^{-1} in case of TC extract. Only the functional group responsible for capping activity was present in the nanoparticles. This result showed that oxidized polyphenols have capped the surface of the TC-AgNPs. The groups such as carbonyl, hydroxyl, and amide presented in the extract may be related to the reduction of metal ions [15,21,28].

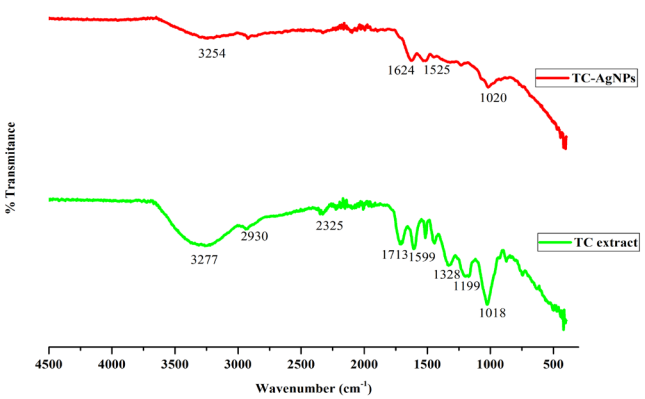


Figure 6: *FTIR spectra for TC-AgNPs and TC extract*

X-ray diffraction analysis (XRD)

The X-ray diffraction analysis was performed to confirm the crystalline nature of the particles, which showed the number of Bragg's reflections that may be indexed based on the face-centred cubic structure of silver.

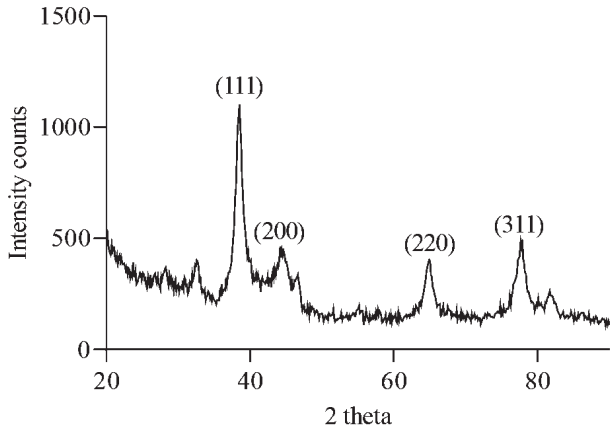


Figure 7: *XRD analysis of the biosynthesized TC-AgNPs*

Figure 7showed the x-ray diffraction (XRD) patterns of dried silver nanoparticles synthesized using *T. chebula* plant extract. There were four distinct peaks at 2θ values of 38.51, 44.18, 64.90, 77.87, with corresponding lattice plane values indexed at (111), (200), (220), and (311). The peaks were the characteristics of the metallic face-centred cubic (FCC) phase of Ag and match with the database of standard (JCPDS Card no. 04-0783) and hence confirmed the crystallinity of the nanoparticles [23]. Similar values were reported earlier [15-28]. Other characteristic peaks may be due to the crystalline nature of the bio-organic phase (capping agent) on the surface of nanoparticles as well as maybe silver oxide due to the partial oxidation of silver. The average crystallite size of the synthesized TC-AgNPs was calculated from the width of all diffraction peaks obtained by the Gaussian fitting of all four peaks using the Debye-Scherrer formula and it was found to be 6.1 nm. The particle size found was slightly low as compared with the literature [28,35,38]. The comparatively smaller value of the size of the nanoparticles can be interpreted with various factors such as pH, temperature, pressure, time, etc [39].

Antioxidant activity (DPPH assay)

Antioxidant activity was determined using a DPPH assay. It is one of the best and most well-known and easy methods for detecting antioxidant activity. Here, the DPPH was scavenged by the hydrogen

atom present in the plant sample. DPPH radical assay was performed for each sample by using quercetin as a standard as described in the standard protocol as mentioned already. The linear regression for the radical scavenging versus concentration was evaluated for determining 50% inhibition of DPPH activity (IC_{50}) values for each sample. The higher value of IC_{50} indicates lower antioxidant activity and also lower IC_{50} value indicates higher antioxidant activity. The corresponding IC_{50}values of the samples were determined using GraphPad Prism version 8.0.1 software.

Furthermore, the aqueous extract of *T. chebula* was also used to synthesize silver nanoparticles. Many authors have reported that the plant contains higher metabolites which are responsible for the metal ion reduction and best capping activity. Hence, the antioxidant property of the silver nanoparticles was evaluated. A range of different concentrations of nanoparticles was prepared by dissolving in 5 % DMSO the plot of percent radical scavenging versus concentration is given below. From the plot of percent radical scavenging versus concentrationof plant extract and AgNPs, the IC_{50} was calculated and compared to the plant extract and AgNPs.

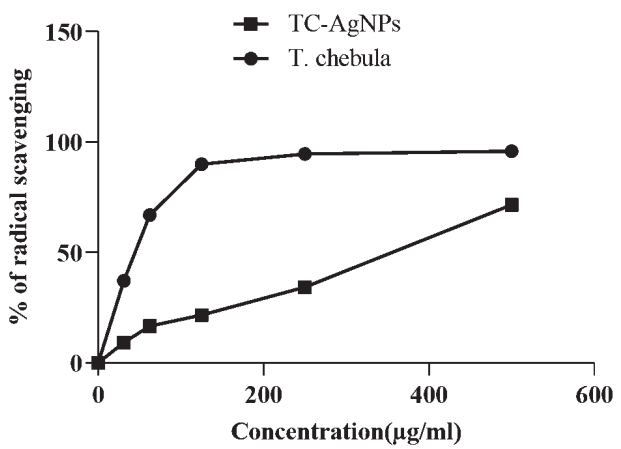


Figure 8:*Percent radical scavenging vs concentration of T. chebula leaf extract and TC-AgNPs*

From the above plot, the value of IC_{50} was found to be 25.433 ± 0.655 µg/mL for *T. chebula,* and 312.8 ± 2.281 µg/mL for TC-AgNPs.The *T. chebula* has got a lower value of IC_{50} as compared to the corresponding value for silver nanoparticles synthesized using *T. chebula*. The IC_{50} values are listed in figure 8.It was found that *T. chebula* leaf extract had higher antioxidant activity as compared with the corresponding silver nanoparticles. More conveniently,the value of IC_{50} of *T. chebula* was well compared with the standard quercetin as shown infigure 9.

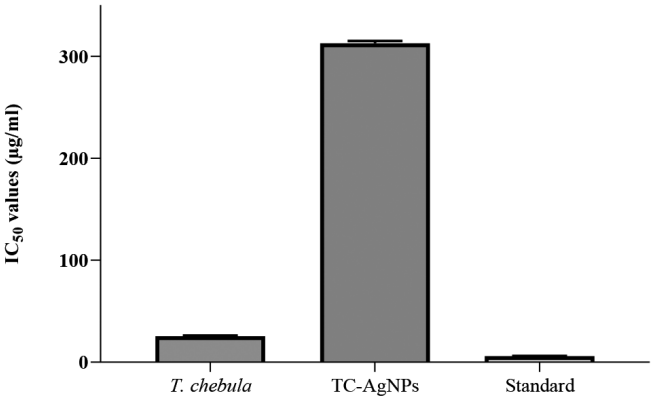


Figure 9:*Antioxidant activity (IC_{50}) of plant extract and AgNPs*

The IC_{50} value for *T. chebula* (25.433 ± 0.655 µg/mL) was found higher than the quantity (11.6± 0.43µg/mL) evaluated by Arya et al. (2012) [31]. The silver nanoparticles synthesized from *T. chebula*leaf extract did not show high antioxidant activity as compared with the methanolic *T. chebula* leaf extract. Such differences in inactivity can be discussed based on the formation of secondary metabolites which are influenced by environmental factors and the geographical location of plant growing.

Antibacterial activity

Plant extract

The antibacterial activity of methanolic TC plant extracts was examined against pathogenic bacteria using the agar well diffusion method mentioned. The positive control used in this case was 1 mg/mL neomycin antibiotic and 50 % DMSO was used as a negative control.

The antibacterial tendency of the plant extracts has been determined based on the zone of inhibition as shown in table 1.

Table 1: *Zone of inhibition for T. chebulaleaf extract and TC-AgNPs*

S.N.	Pathogens	Zone of inhibition(mm)		
		PC	RTC	NC
1.	*Staphylococcus aureus* (ATCC 25293)	22	14	0
2.	*Acinetobacter baumannii* (ATCC 19606)	20	12	0
3.	*Salmonella typhi* (ATCC 14028)	16	0	0
4.	*Escherichia coli* (ATCC 3292)	16	0	0
5.	*Klebsiella pneumoniae* (ATCC 700603)	18	0	0
6.	*Shigella sonnei* (ATCC 25931)	25	22	0

PC-Positive control, **RTC**-*T. chebula* methanolic extract, **NC**-Negative control

Methanolic leaf extract of *T. chebula* showed some inhibition in the bacterial growth for gram-positive bacteria *Staphylococcus aureus* (ATCC 25293), and gram-negative bacteria *Acinetobacter baumannii* (ATCC 19606), *Shigella sonnei* (ATCC 25931). However, the value of the zone of inhibition was not so high so further work was not performed to test the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC).

Silver nanoparticles

Antibacterial activity of TC-AgNPs was examined against pathogenic bacteria using the agar welldiffusion method mentioned. The positive control used in this case was 1 mg/mL neomycin antibiotic and 5 % DMSO was used as a negative control. The antibacterial tendency of the plant extracts has been determined based on the zone of inhibition as shown in Table 2. TC-AgNPs showed significant antibacterial activity towards the tested pathogenic microorganisms. However, the value of the zone of inhibition was not so high so further work was not performed to test the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC).The exact mechanism for the antibacterial activity of silver nanoparticles is not well known. But theoretically, many studies reported that nanoparticles bind to the bacterial membrane, invade the cell and cause the proton motive force which leads to the distraction of bacterial cells by forming pores on the bacterial cell wall and finally inhibits the growth. The silver nanoparticles were most active against gram-positive bacteria as compared with gram-negative bacteria [40].

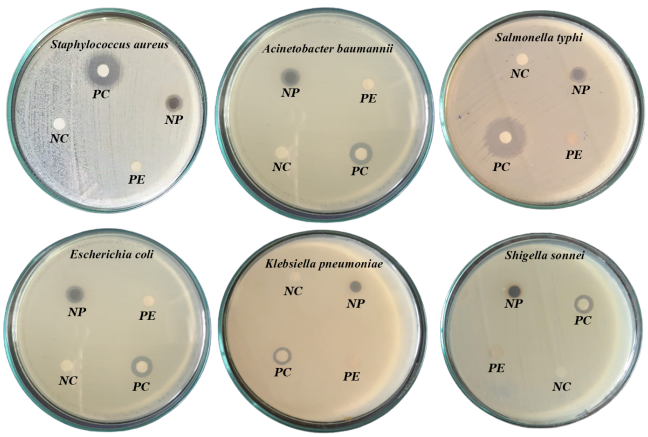


Figure 10: *Antibacterial activity of TC-AgNPs (NP) and T. chebula leaf extract (PE)*

Table 2: *Zone of inhibition for TC-AgNPs*

S.N.	Pathogens	Zone of inhibition(mm)			
		PC	TC-AgNPs	PE	NC
1.	*Staphylococcus aureus* (ATCC 25293)	15	9	0	0
2.	*Acinetobacter baumannii* (ATCC 19606)	11	9	0	0
3.	*Salmonella typhi* (ATCC 14028)	18	8	0	0
4.	*Escherichia coli* (ATCC 3292)	12	7	0	0
5.	*Klebsiella pneumoniae* (ATCC 700603)	9	0	0	0
6.	*Shigella sonnei* (ATCC 25931)	12	0	0	0

PC-Positive control, **PE**-Aqueous*T. chebula* extract, **TC-AgNPs**-Silver nanoparticles using*T. chebula* aqueous extract, **NC**-Negative control

The green synthesized silver nanoparticles have shown promising antimicrobial activities [28,35]. In some previously reported results, it is mentioned that the high antimicrobial activities of green synthesized silver nanoparticles over the other Physico-chemical methods [41]. It is due to the different capping and

reducing behaviour of secondary metabolites found in the plant extract.

Brine shrimp lethality bioassay

A brine shrimp assay was performed to test the toxic nature of the plant extracts and green synthesized TC-AgNPs. Ten shrimp larvae were allowed to expose to each varied concentration of the sample. After 24 h, the alive larvae were counted properly, and finally, the percentage mortality was observed as shown in table 3.

Table 3:*Toxicity test of plant extract and green synthesized TC-AgNPs*

Sample	Concentration (µg/mL)	Nauplii taken	Alive nauplii	Dead nauplii	% Mortality
T. chebula	10	10	9	1	20
	100	10	7	3	30
	1000	10	6	4	50
TC-AgNPs	10	10	9	1	20
	100	10	7	3	30
	1000	10	6	4	60

Also, the lethal concentration that kills 50% of the exposed population of *A. salina* (LC_{50}) was calculated using % mortality as shown in 4.

Table 4: *Calculation of LC_{50} value of plant extract and green synthesized TC-AgNPs*

Sample	*T. chebula*			TC-AgNPs		
Z	10	100	1000	10	100	1000
x	1	2	3	1	2	3
y	4.16	4.48	5	4.16	4.48	5.25
xy	4.16	8.96	15	4.16	8.96	15.75
x^2	1	4	9	1	4	9
$\sum x$		6			6	
$\sum y$		13.64			13.89	
$\sum xy$		28.12			28,87	
$\sum x^2$		14			14	
β		0.42			0.55	
α		3.71			3.54	
X		3.07			2.68	
LC_{50}	1177.61 ± 4.68			477.53 ± 0.68		

The methanolic extract of *T.chebula* was found less toxic to the shrimps with a value of 1177.61 ± 4.68 µg/mL. However, the TC-AgNPs were moderately toxic to the shrimps. Further, it was found that the degree of lethality was found to be directly proportional to the concentration of the sample used. That is higher the value of concentration, the higher was the mortality rate. Although this method does not provide any adequate information regarding the mechanism of toxic action, it is a very useful method for the assessment of the toxic potential of various plant extracts. This method provides preliminary screening data that can be backed up by more specific bioassays [42]. The exact mechanism of the lethality tendency of the silver nanoparticles is not well studied yet. Some of the research studies have believed that nanoparticles aggregates to the elevated levels of the gut that were filled with particles showed significant mortality within 24 h exposure [43].

Conclusions

Biogenic method was adopted for the synthesis of silver nanoparticles using *Terminalia chebula* Retz. Leaf extract and the method wasfound to be very efficient in terms of reaction time and stability. It was found that plants with high phenolic and flavonoid contents can be considered not only as their potency against antioxidant activity but also with strong metal ion reducers. A high value of flavonoids and phenols could have supported the fast and easy reduction of silver ions and their stabilization. Characterization of silver nanoparticles can be well illustrated by UV-visible spectroscopy, FTIR, and XRD analysis. UV-visible spectra suggested the formation of silver nanoparticles showing surface plasmon resonance with the λ_{max} (420 nm). FTIR analysis confirmed the bio-reduction of Ag^{+} ions to AgNPs by various functional groups and their role in capping activity. The biosynthesized TC-AgNPs were crystalline as evident from XRD analysis. The average particle size of the biosynthesized silver nanoparticles was 6.1 nm. TC-AgNPs exhibited average antioxidant and strong antibacterial activity against four pathogenic bacteria like *Staphylococcus aureus*, *Acinetobacter baumannii*, *Salmonella typhi*, and *Escherichia coli* with moderate cytotoxic activity (LC_{50} 477.53±0.68 µg/mL) against brine shrimps in a dose-dependent manner.Hence, AgNPs synthesized from this approach have potential applications in the fields of medicine, especially in drug delivery.

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