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RESEARCH ARTICLE

Green synthesis of silver nanoparticles from *Verbena officinalis* L.: Characterization and antibacterial efficacy

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Abstract

In recent times, attention has been paid to the green synthesis of nanoparticles using plant extracts due to their eco-friendly, low-cost and easy modes. In the present research, silver nanoparticles (AgNPs) were synthesized using methanolic root extract of *Verbena officinalis* L. The AgNPs were tested for antibacterial activity against *Escherichia coli* ATCC-O157 H: 7 and *Staphylococcus aureus* ATCC-25923 using the disc diffusion method and minimum inhibitory concentration (MIC) determination. The AgNPs formation was initially observed by the color change and further verified using UV-Vis spectrophotometer, Fourier Transform Infra-red (FT-IR) and X-Ray crystallography (XRD). The color change from yellow to brown in the reaction mixture was an indication of the synthesis of AgNPs. Further confirmation was established at 434 nm in UV-Vis spectroscopy. The FTIR analysis displayed bands analogous to functional groups of the plant's phytochemicals that were accountable for reducing and capping of AgNPs. The AgNPs analysis by XRD validated the crystallinityof AgNPs at 2θ angles of 38.00°, 44.16°, 64.40° and 77.33° harmonizing to planes at (111), (200), (220) and (311), respectively and established their average size (22.67 nm). The AgNPs exhibited strong antibacterial potential against the tested pathogenic bacteria. The AgNPsexhibited significantly (p<0.001) better antibacterial effect against *S. aureus* than *E. coli*. The smallest MIC of 10.42 µg/mL was observed against *S. aureus*. These outcomes demonstrated the efficacy of components of the *V. officinalis* root extract in the significant enrichment of the toxicity of the AgNPs against the bacterial pathogens.

Keywords: antibiotic resistance; *Escherichia coli*; phyto-synthesis; silver nanoparticles; *Staphylococcus aureus; Verbena officinalis*

Introduction

Infectious diseases caused by microorganisms are crucial health hazards in humans all over the world. Numerous conventional drugs have been in use to fight infectious communicable diseases. Despite the availability of many conventional antibiotics and drugs to treat infections caused by microbes, resistance has been developed by the pathogenic microorganisms and this has been a startling problem for several years (1). According to the report of Antimicrobial Resistance Collaborators (2), about 1.27 million people lost their lives in 2019 due to antimicrobial resistance. *Staphylococcus aureus* and *Escherichia coli* are the two most antimicrobial-resistant bacterial pathogens (3, 4). Ethiopia, in Africa, is also facing the menace of antibacterial resistance due to the emergence of drug-resistant bacterial isolates including *E. coli*, *Salmonella* spp. and *Staphylococci* spp. (5, 6). Apart from their failure to fight microbial pathogens, synthetic drugs or antibiotics also cause side effects on humans (7). Therefore, advanced research on the development of novel alternative antimicrobial agents that are safe and eco-friendly is in dire need to fight microbes resistant to synthetic drugs.

The jeopardy of antimicrobial resistance and side effects of conventional drugs have necessitated alternative therapy for microbial infections from plant resources along with nanoparticles. The nanoparticles have been known to exhibit enhanced bioactivities due to their unique properties and relatively higher surface area to volume ratio and small size than their bulk precursors (8, 9). Among the nanoparticles, silver nanoparticles (AgNPs) have gained much consideration as silver has been substantiated to possess antibacterial efficacy (10) and they are less toxic to human cells (11). Thus, endeavors have been made to establish the antibacterial effects of bio-synthesized nanoparticles and the synergistic effect of both the metal and the plant extracts (12-14). Functional groups of secondary metabolites in the extracts reduce silver salt (Ag^+) to (Ag) and cap the phyto-synthesized nanoparticles (15). Above all, phyto-synthesis of nanoparticles is highly preferred as this method is cost-effective, efficient, eco-friendly and involves comparatively a smaller amount of energy than physical

and chemical approaches (16). Thus, the current study was designed to synthesize AgNPs using methanolic root extract of *V. officinalis* L., which belongs to the Verbenaceae family and to investigate their antibacterial activity. *V. officinalis* is a hard and herbaceous perennial plant distributed in North and Central Africa, Asia, America, Europe and Australia. Previous studies show that crude extract of *V. officinalis* is used to treat tonsillitis and diarrhea (17) and shows antibacterial property (18). The plant is used in treating upper respiratory, digestive and urinary tract infections (17, 19-24). Phytochemical studies of *V. officinalis* showed the occurrence of sterols (α -sitosterol, β -sitosterol, limonene, cineole and carvone), terpenoids (rosmanol, isorosmanol, kaempferol, luteolin and apigenin), flavonoids (scutellarein and pedalitin) and phenolic acids (chlorogenic acid, rosmarinic acid and protocatechuic acid) (25–31). Biological activities like antibacterial (32, 30), antioxidant (27), anti-inflammatory (33), neuroprotective activity (34), anticancer activity (35, 36) and wound healing property (37) of *V. officinalis* were investigated. Sanchooli et al. (38) also reported that AgNPs synthesized from leaf extracts of *V. officinalis* demonstrated antibacterial activity. However, there is no such data on phyto-synthesis of AgNPs using root extracts of this plant species to evaluate its antimicrobial property. Here, we hypothesized that AgNPs synthesized by using root extract of *V. officinalis* also show antibacterial activity. Hence, we attempted to investigate the antibacterial effects of AgNPs synthesized using methanol extract of *V. officinalis* root against *S. aureus* and *E. coli*.

Materials and Methods

Chemicals

Ampicillin, methanol, Muller-Hinton agar, nutrient agar, silver nitrate, Bromocresol green, Dimethyl sulfoxide, Folin's reagent, Vanillin reagent, Hexayanoferrate (III) solution, Acetic anhydride, Linalool solution, Baljet reagent, Dragendorff's reagent

Preparation of extract

Newly harvested and healthy *V. officinalis* roots were collected from *Dugda* District of *Arsi* Zone, *Oromia* region, Ethiopia. The specimen was authenticated by the Botanists of the department and deposited in the Herbarium of the University. The collected root part was washed to remove soil particles and then rinsed with distilled water. The sample was dried in an oven (Bluefic, India) at 40 °C, 96 h. The dried roots were powdered with a mortar and pestle. The powdered material (50 g) was macerated in 250 mL methanol (98%) and left on an electrical shaker (SGS, India) for 48 hours for extraction. The mixture was filtered by Whatman No. 1 paper and the filtrate was then kept at 4°C in dark for the synthesis of nanoparticles.

Qualitative tests for secondary compounds

The methanolic extract of *V. officinalis* root was subjected to qualitative tests to detect various secondary compounds viz. alkaloids, steroids, flavonoids, saponins, tannins, terpenoids and glycosides following the method of Harborne (39).

Silver NPs' synthesis

The synthesis of AgNPs was accomplished according to the technique adopted by Yugandhar et al. (40) with minor modifications. Silver nitrate (1 mM) solution was made by adding 0.017 g of $AgNO_3$ in distilled water (100 mL) to prepare the precursor for the silver. To 95 mL of 1 mM silver nitrate solution, 5 mL of the extract was added and the mixture was left for 24 h on a shaker at room temperature. Then, the reaction mixture was centrifuged (12,000 rpm; 15 min) to collect the silver NPs. Consequently, the pellets were obtained after elimination of the supernatant. The pellets were then washed twice in double-distilled water and dried in an oven at 80 °C. After drying, the synthesized AgNPs were used for further characterization and antibacterial activity.

Characterization of phyto-synthesized AgNPs

Visual observation and UV-Visible spectrophotometer

The primary confirmation of the synthesis of the AgNPs was achieved by observing the changes in color of the medium. The reduction of silver ions by plant extract into AgNPs was monitored by UV-Visible spectroscopy (Lambda -9-UV-Visible Spectrophotometer, Perkin Elmer, USA), functioning at the range of 300 nm and 700 nm resolution at room temperature at different time periods, i.e., at 10, 30, 90 minutes and 24 h. Silver nitrate (1 mM) was used as a blank.

XRD characterization

The XRD data (Shimadzu XRD - PXRD-7000) of the phyto-synthesized AgNPs was documented in the range of 30 to 80° (2θ) with the use of CuKa radiation. The energy used was 8.04 keV, with 1.54 Å wavelength, 40 kV voltage and 25 mA current. The size of the crystals was measured by the equation of Scherer as follows.

$$D = (0.9X\lambda) / \beta X Cos\theta$$

Where,

D - Crystal size (Average), β - Line broadening of radians, θ - Bragg's angle, λ - Wavelength of X-Ray.

FT-IR analysis for characterization

The FT-IR (Perkin Elmer Frontier FTIR C109832) was carried out to analyze molecular functional vibrations that were recognized and assigned to define the chemical groups involved in reducing, capping and stabilizing the nanoparticles. The FT-IR spectrum was recorded at 2 cm^{-1} resolution in the frequency at 4000 cm^{-1} to 400 cm^{-1} infra-red range. To make infrared analysis appropriate, FT-IR grade potassium bromide (KBr, 100 mg) pellet was mixed with dried AgNPs (1 mg) at 1:100 ratio (AgNps:KBr).

Evaluation of antibacterial activity

Antibacterial susceptibility test to AgNPs

Bacterial strains, *S. aureus* (ATCC 25923) and *E. coli* (ATCC O157 H:7) were procured from the Ethiopian Public Health Institute, Ethiopia. Nutrient agar and Petri dishes were autoclaved (Danfoss, Poland) before the antibacterial test.

Uniform samples of both bacteria were inoculated on standard media (nutrient agar) using a disposable spreader. After inoculation, incubation was done for 24 h at 37°C. They were then transferred to nutrient media for sub-culturing and after 24 h incubation, it was used for the antibacterial test. The antibacterial efficacy of phyto-synthesized AgNPs against the selected bacteria was evaluated using standard method (41). The density of sub-cultured bacterial strains was made equivalent to the McFarland (0.5) standard (1.0×10^7CFU/mL). To prepare MHA medium, MHA (38 g) was mixed with 1 L of distilled water and autoclaved for 20 min at 121°C. This MHA medium was solidified in Petri dishes and kept in a sterile condition. Then, 100 µL of pre-grown bacterial cultures were uniformly inoculated onto the MHA plates and kept for drying. The sterile paper discs with 6 mm diameter were permeated by AgNPs (20 µL) of various concentrations (25 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL) and placed on the Petri dishes and incubated at 37°C. After 24 h, the effect of AgNPs on the bacterial strains was evaluated by calculating the zone of inhibition. The positive control used was Ampicillin (30 µg/mL), the standard reference antibiotic and the negative control was distilled water. Triplicate tests were performed and the mean ±standard deviation was calculated for the obtained values.

Determination of Minimum Inhibitory Concentration (MIC) of AgNPs

The MIC of the Ag nanoparticles was evaluated by the standard two-fold serial dilution method (41). Briefly, the inoculums of 1.0×10^7CFU/mL of the test microbes were prepared from 24 h broth cultures in sterile test tubes. The AgNPs in the range of 25 to 1.5 µg/mL concentrations were used for the determination of MIC. Firstly, 25 µg of the AgNPs was added to the first tube comprising 2 mL of MHA broth and then 1 mL of it was transported to the second tube containing 1 mL of MHA medium. The dilution was prepared till the concentration of the final tube attained 1.5 µg/mL. Then, the tubes were added with 100 µL of bacterial test strains. Lastly, the tubes were mixed properly and incubated at 37°C. After overnight incubation, the least concentration with no growth (no turbidity) was marked as the MIC value. The MIC value was expressed as µg/mL.

Analysis of data

The analysis of statistics was carried out by SPSS, version 20. The general linear model was employed to check the impact of the main effects (bacterial strain and AgNPs concentrations) and their interactions. Differences between mean zones of inhibition were considered significant at $p<0.05$.

Results and Discussion

Qualitative test

The preliminary phytochemical screening revealed that flavonoids, alkaloids, saponins, steroids, tannins and terpenoids were found in the methanolic extract of *V. officinalis* root, whereas glycosides were absent. Although not from the root, previously, researchers (42,43) have reported the occurrence of the above said compounds in *V. officinalis* aerial parts.

Characterization of AgNPs

Visual observation

Upon extraction, the methanolic root extract of *V. officinalis* was yellow (Fig. 1B). After mixing with the colorless $AgNO_3$ solution (Fig. 1A), the mixture of extract and the precursor solution became brown (Fig. 1C) after 24 h. This change in color suggests that Ag^+ is reduced to silver (Ag) nanoparticles and self-assembles into colloidal particles (AgNPs) (44) due to reducing properties of phytochemicals (e.g., phenolics and terpenoids) that are found in the plant extract. The formation of brown color has been ascribed to the phenomenon of surface plasmon resonance (SPR) (12).

Characterization of AgNPs by UV-Vis Spectrophotometer

A UV-Vis Spectrophotometer was used to measure the absorption spectra of synthesized silver NPs at various intervals of time. The variation in the absorption of the mixed precursor and extract solutions at various intervals of time is depicted in Fig. 2. The optical density of synthesized AgNPs displayed a gradual increment in the absorption. The intensity of the maximum absorption peak observed was 434 nm after incubation for 24 h, which shows the total reduction of $AgNO_3$ to AgNPs. Here, the

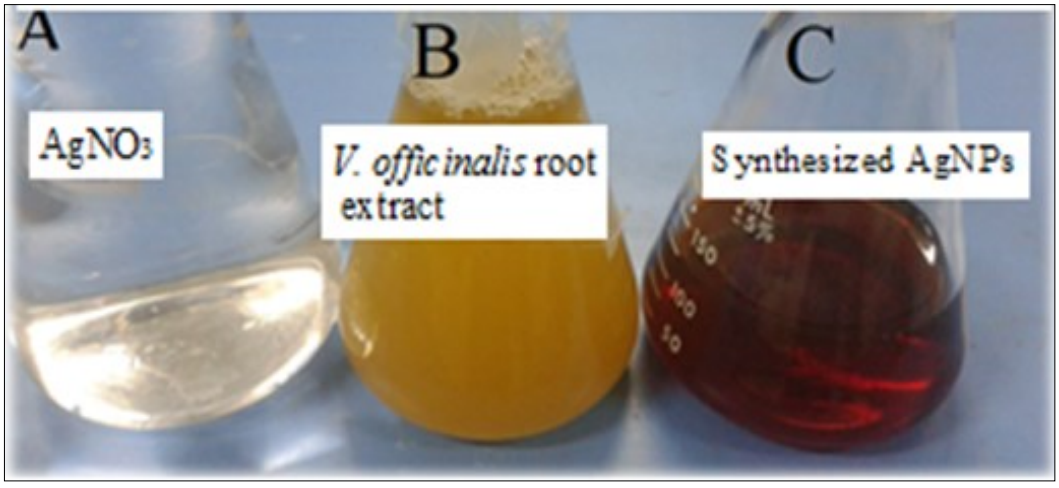

Fig. 1. Silver nitrate ($AgNO_3$) solution (A), methanolic root extract of *V. officinalis* (B) and reaction mixture of $AgNO_3$ and the extract (C).

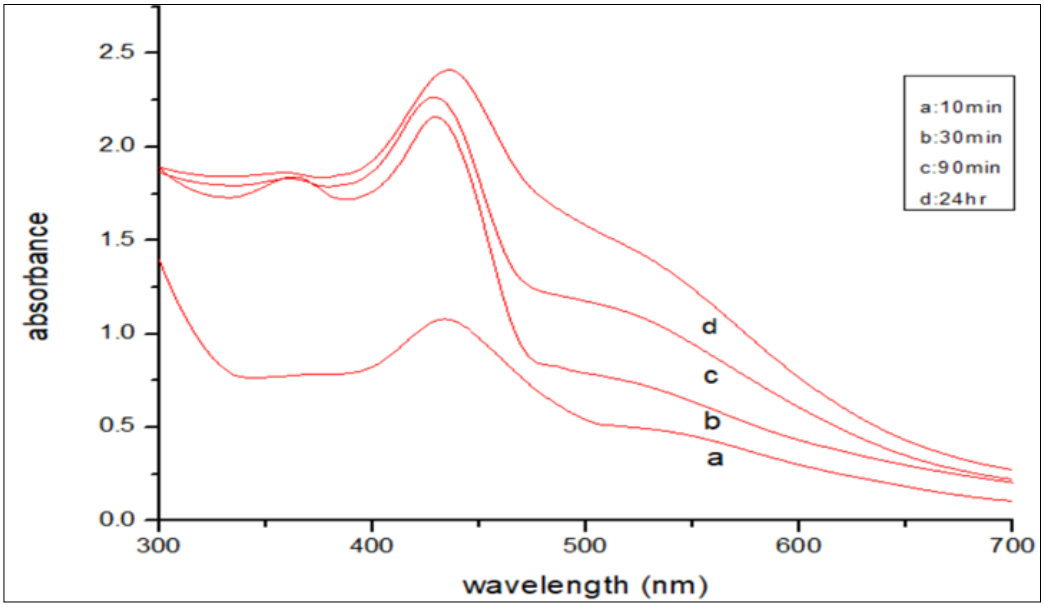

Fig. 2. Absorption spectra in UV-Vis for the synthesized Ag nanoparticles at different intervals of time. Note: curves a, b, c and d correspond to 10, 30, 90 min and 24 hours, respectively.

size and shape of the nanoparticles determine the perceptible spectral characteristics of AgNPs, which are based on the SPR (45). The SPR or absorption band of *V. officinalis* mediated AgNPs exhibited an absorption peak at 434 nm, which relates to the absorbance of silver NPs. A previous study (12) reported that SPR of AgNPs of leaf extract of *Moringa oleifera* showed a peak centered near 440 nm, which is closer to our result. A previous work on synthesis of AgNPs using *V. officinalis* leaf from Iran showed that the maximum absorption observed was around 420 nm (38). Some previous studies showed the maximum absorbance for AgNPs was at 470 nm (46) and 460 nm (47). This variation could be due to the varying extract constituents obtained from different sources to react with silver nitrate, which leads to different interventions with absorption signs of the SPR (45).

Characterization of AgNPs by FT-IR

The FT-IR analysis was carried out to provide data on probable functional groups of plant components found on synthesized AgNPs' surface (Fig. 3). The spectra obtained from FT-IR were recorded for AgNPs from 4000 cm^{-1} - 400 cm^{-1} at 4 cm^{-1} resolution in KBr pellets. The absorption peaks at different locations observed were 3446, 2936, 2922, 1740, 1627 and 1031 cm^{-1} associated with various functional groups in phytomolecules. The absorption band 3446 cm^{-1} is associated with OH stretching vibrations of phenolics and also attributed to amino acids with N-H stretch (48). The absorption bands 2936 and 2922 cm^{-1} are ascribed to C-H alkenes' and carboxylic acids' stretch, respectively (49, 44). The peak at 1627 cm^{-1} is attributed to the compounds with carboxylic, aldehydes, esters or ketone groups with C=C stretching (50). The peak at 1740 cm^{-1} is ascribed to C=O carboxylic acids and the band at 1031 cm^{-1} is assigned to C-O stretching vibrations of alcoholic groups (51). The characterization of AgNPs by the FT-IR suggested that the Ag nanoparticle formation by Ag salt's reduction, capping and stabilization might be attributed to phenolic compounds' oxidation (48). In the current study, qualitative screening of the methanolic root extract of *V. officinalis* exhibited the occurrence of proteins, flavonoids and phenolics that might have contributed to the formation of AgNPs from its precursor.

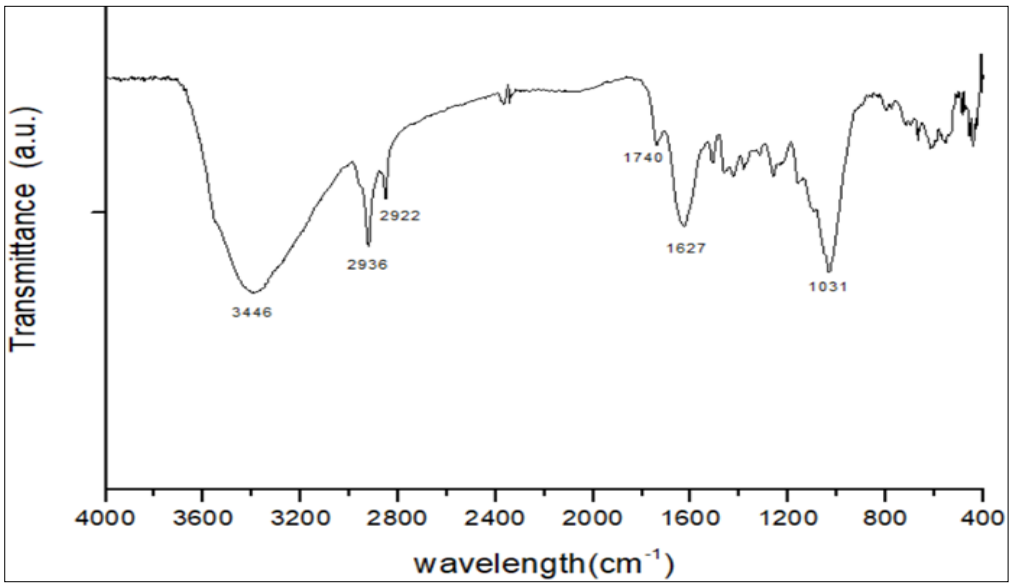

Fig. 3. The spectral data of silver NPs in FTIR.

Characterization of AgNPs by XRD

In this study, XRD was employed to identify the crystal nature of the phyto-synthesized Ag nanoparticles in the 30 -80° range at 2θ angles. Fig. 4 represents the X-ray diffraction pattern of AgNP colloids by reaction between aqueous Ag salt and *V. officinalis* root extract. Four distinct peaks of diffraction of silver NPs were detected at 38.00°, 44.16°, 64.40° and 77.33° agreeing to planes at Bragg's reflections (111), (200), (220) and (311), respectively, that are reflections of metallic Ag's face-centered cubic (FCC) structure. The detected planes suggest that the synthesized AgNPs' nature was crystalline. The remaining unassigned peaks at the angle of 2θ might be attributed to impurities in the sample or ascribed to crystals of organic contents (52-54). This result is in consonance with the results of other researchers (53, 55). The equation of Debye -Scherer was employed to measure the synthesized AgNPs' average crystal size from the FWHMs values of the peaks of Bragg diffraction. The size of the crystallites in different planes of Ag are given in Table 1 and the mean value of all peaks was 22.67 nm. The findings of the current study are in line with work done earlier (52).

Table. 1. The size of synthesized silver NPs crystals

Peak position (2θ)	FWHM*	Crystallite size (nm)
16.05617	0.1816	44.17905148
20.76533	0.17738	45.53238153
32.97829	0.30826	26.87673648
37.92958	0.72732	11.54961839
42.2934	0.22154	38.44851978
43.71474	0.61508	13.91625359
55.20086	17.0826	0.524770324
74.79076	26.91787	0.371484197
Average crystallite size (nm)		**22.67485197**

* FWHM = Full Width at Half Maximum

The bactericidal effect of synthesized AgNPs

The current study emphasized the effect of AgNPs against *E. coli* and *S. aureus*, which cause infection of soft tissues, bacteremia and deadly disease pneumonia, but have been reported to be resistant to methicillin (56). *E. coli* (O157 H:7 strain) is known to cause communicable diseases in human beings (57) and this strain has been reported to show resistance to antibiotics (58). The findings of the present study showed that the main effects, i.e., bacterial species and AgNPs' concentration, had significant effect (p<0.001) on the measured zone of inhibition. Likewise, the inhibition zone was also significantly (p=0.028) affected by the interaction between bacterial species type and concentration of silver NPs (Fig. 5). Since no growth inhibition was seen in a negative control (distilled water) values are not shown in Fig. 5. However, the growths of both bacterial species were inhibited by AgNPs in a concentration dependent manner. Comparison between the two bacterial species showed that Gram (+) *S. aureus* was much sensitive to AgNPs than Gram (-) *E. coli* (Fig. 5, 6). Generally, Gram negative bacteria are more susceptible to antibacterial agents as they have thinner peptidoglycan layer than the Gram-positive bacteria (59). On the contrary, our results showed that Gram negative *E. coli* is less prone to AgNPs than *S. aureus,* which is consistent with the previous result (60). This might be due to additional outer membrane and other cell wall thickness factors of Gram-negative bacteria (61). The MIC of silver NPs of *V. officinalis* against Gram (-) *E. coli* and Gram (+) *S. aureus* was also investigated using the serial dilution method after confirming the bactericidal effects in the disc diffusion technique. The MIC of the AgNPs was found to be lower (10.42 µg/mL) when tested against *S. aureus* (16.67 µg/mL) than when tested against *E. coli.*

The antibacterial effect of AgNPs towards *E. coli* and *S. aureus* might be due to altered permeability of the cell membrane of the bacteria (62), leakage of reducing sugar and proteins from the bacteria (63, 64) and formation of free radicals by the AgNPs that cause cell death (65). Several studies (66, 67, 45, 50, 61) reported that important bacterial enzymes are inactivated by silver ions (Ag^+) produced by oxidation of AgNPs on their surface in aqueous medium. It is well known that there is electrostatic attraction between positively charged silver (Ag^+) and negatively charged bacterial cells (68). These Ag^+ generated by AgNPs oxidation possess high affinity towards sulfur (S) or phosphorus (P) components of the

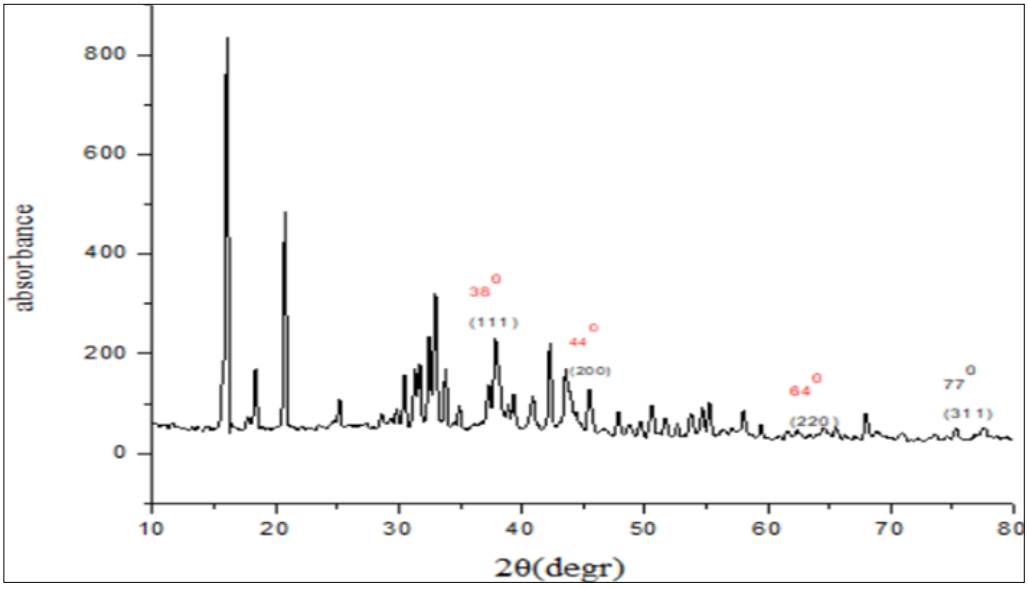

Fig. 4. Spectra of XRD of synthesized silver NPs.

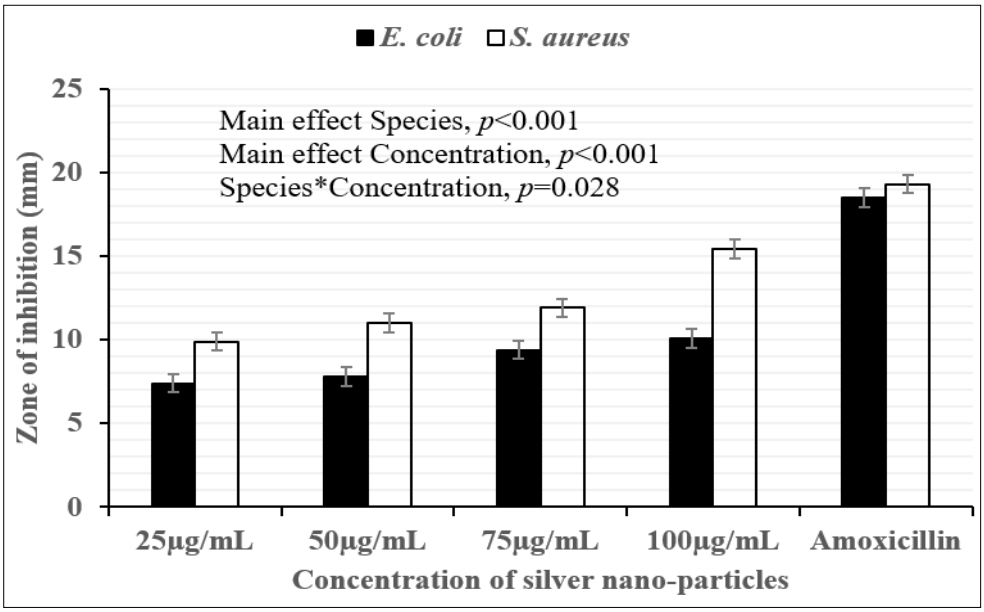

Fig. 5. Antibacterial activity of the synthesized AgNPs.

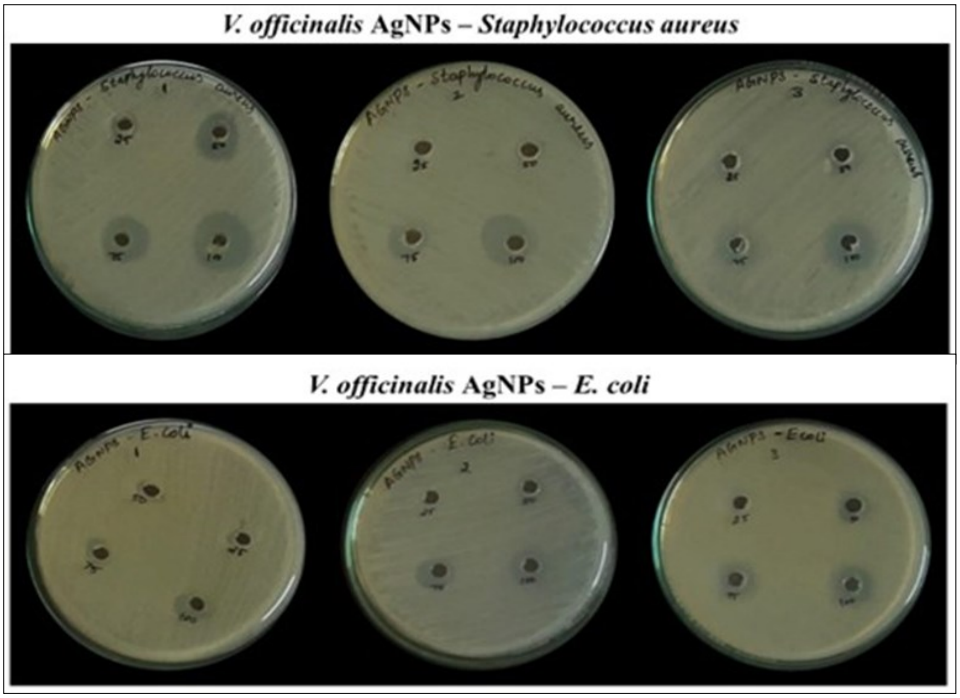

Fig. 6. Antibacterial activity of the synthesized AgNPs.

bacterial plasma membrane. After attraction to S and P, Ag^{+} will associate with proteins with S and other components like DNA containing P. These alterations in bacterial membrane proteins and DNA will adversely influence the cell metabolic processes such as cellular respiration and cell division, which eventually lead to cell death (69, 70, 50). Besides the size and surface area, the AgNPs were an obvious reason for their antimicrobial property that could enable penetration and permeation of AgNPs into cell walls of bacteria (71). The small sized nanoparticles with large surface to volume ratio afford a greater level of interaction with microbes for antibacterial effect (72). The nanoparticles produced in our study were found to be small in size (22.67 nm), which must have contributed to the antibacterial action towards the tested pathogenic bacteria.

Conclusion

The current study revealed the potential of *V. officinalis* root extract for the synthesis, capping and stability of AgNPs. The phyto-synthesized AgNPs were characterized successfully with UV-Vis spectra, FT-IR and XRD techniques. The green synthesized AgNPs showed maximum absorption peak of 434 nm in UV-Vis spectra. The FT-IR analysis provided information functional groups of the phytochemicals of *V. officinalis*. The X-ray crystallographic study exhibited four different peaks of diffraction at 38.00°, 44.16°, 64.40° and 77.33° at Bragg's reflections (111), (200), (220) and (311). A potent antibacterial activity of the AgNPs against *E. coli* and *S. aureus* was observed. The AgNPs synthesized displayed concentration dependent antibacterial activity against the tested pathogens. Hence, this work can be reflected as an endeavor for further research in Ethiopia on nanoparticles synthesized by plant extracts to combat a broad spectrum of pathogenic microorganisms.

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Authors' contributions

SJM conceptualized the work, drafted the manuscript and edited the paper. SA carried out the experimental studies and drafted the manuscript. MCE edited the manuscript and performed the statistical analysis. JPR participated in the design of the paper and coordination. SB participated in conceptualization of the work. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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