

BIO-INSPIRED GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *ACOKANTHERA SCHIMPERI* AND ASSESSMENT OF THEIR ANTI-BACTERIAL ACTIVITY AGAINST MDR BACTERIA

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ABSTRACT. Increasing multi drug resistance in bacteria against currently available antibacterial agents, toxicity and allergies are considered as serious health problem. Hence there is an urgent need for the antibacterial agents. This study is the first in its kind which focuses a simple green synthesis of silver nanoparticles (AS-AgNPs) using *Acokanthera schimperi* aqueous leaf extracts to evaluate *in vitro* antibacterial activity. The AS-AgNPs were confirmed by using various techniques such as UV-Vis, FTIR, XRD, DLS, TEM, SEM-EDX and evaluation of their antibacterial activity against multidrug-resistant bacteria *S. aureus*, *S. mutans*, and *K. pneumoniae*. TEM analysis revealed that AS-AgNPs are spherical, poly dispersed with an average size of 20.4 nm. XRD analysis displayed that its nature is crystalline. FTIR analysis stated that presence of different functional groups in aqueous leaf extract of *A. schimperi* act as reducing and capping agents for green synthesis of AS-AgNPs. AS-Ag NPs has shown an enhanced broad spectrum *in vitro* antibacterial activity against tested *S. mutans* with zone of inhibition 12.3-17.4 mm, at 50 to 100 µg/mL compared to AS-AQ extract had shown moderate antibacterial activity against *S. mutans* (9.1-10.7 mm) at 50-100 µg/mL.

KEY WORDS: *Acokanthera schimperi*, Multidrug resistance bacteria, Green synthesis, Silver nanoparticles, Antibacterial activity

INTRODUCTION

Over the past few decades, scientific community has attained interest in the multi-disciplinary field of science and technology; the developments of different approaches for the synthesis of nanomaterials which has been rapidly increased. Today, nanotechnology is starting to open up new possibilities to changing health care, electronic devices, energy storage, textile industries and cosmetics to make human living standards smarter and more sustainable [1]. Nanotechnology is a rapidly emerging field of study that deals with the production and manipulation of nano particles with tunable physicochemical properties of 1–100 nm in size in any dimension [2]. Nanoparticles are continually gaining great attention due to their unique features, which includes high surface-to-volume ratio, high surface energy and distinct mechanical, thermal, electrical, magnetic, and optical characteristics that allows potential application in wide range of disciplines, such as electronics, biology, chemistry, physics, food science and medicine [3, 4]. Bio-nanotechnology is the cutting-edge area which bridges the biological sciences moreover; nanotechnology has a remarkable application in the devolvement of nano-medicines such as; novel nano-antibiotics, nano-vaccine, disinfectants, nano-ointments nano-bandages and dressing materials [5].

Metal and metal-oxide NPs such as silver (Ag), copper (Cu), platinum (Pt), zinc oxide (ZnO) and titanium dioxide (TiO₂) are used in the formulations of nano-gels for effective treatment of infectious diseases [6]. The majority of the above mentioned, environmentally friendly approaches address difficulties like stability, crystal formation, and particle

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aggregation. Among the most important types of nanoparticles, metallic nanoparticles, silver nanoparticles (AgNPs) there is a wide range of applications and are marketable, because they exhibit different optical, biomedical and electrical properties [7]. Indeed, Silver products have been used for centuries to prevent and treat diseases, particularly infections, due to their inhibitory and bactericidal attributes [8-10].

Plants have bioactive compound which have diversified pharmacophore for biological activity and acts as a reducing, capping and stabilizing agent for the synthesis of metal and metal oxide nanoparticles by controlling size, shape, stability and biocompatibility [11]. *Alburae et al.* [12], reported that, the green synthesized silver nanoparticles using seed extracts of *Lasiurus scindicus* and *Panicum turgidum* with spherical shape have shown significant broad spectrum antibacterial activity against Gram positive bacteria *S. aureus* and *S. mutans*, and Gram-negative bacteria *E. coli* and *K. pneumonia*. Ghasemi *et al.* [13], reported that the green synthesized silver nanoparticles from aqueous leaf extract of *Rubus discolor* with spherical shape and average size of 37 nm has shown antibacterial activity against *P. aeruginosa* and *E. coli* with less minimum inhibitory concentration (MIC) value.

The leaves of *Acokanthera schimperi* from *Apocynaceae* family are traditionally used in Ethiopia to treat antiseptic, tonsillitis, skin diseases, and snake bites for its antimicrobial and anti-inflammatory properties. Phytochemical studies reveal that *Acokanthera schimperi* has a number of secondary metabolites and were identified with maximum presence of flavonoids, terpenoids, phenolic compounds, tannins, saponins, glycosides, and phytosterol, and exhibited significance *in vitro* antioxidant activities [14]. Based on the researchers' review of existing literature, no studies have been conducted on the aqueous extract of the plant beyond initial screenings. While many medicinal plants are being explored for the development of green synthesized silver nanoparticles as antibacterial agents, there has yet to be any report on the green synthesis of silver (Ag) nanoparticles from *Acokanthera schimperi* or their antibacterial activity. Therefore, this study aims to synthesize silver nanoparticles using aqueous extracts from dried leaves of *Acokanthera schimperi*, characterize these nanoparticles through spectroscopic and microscopic methods, and evaluate their *in vitro* antibacterial activity against two Gram-positive bacteria, *S. aureus* and *S. mutans*, as well as one Gram-negative bacterium, *K. pneumoniae*.

MATERIALS AND METHODS

Chemicals

All reagents and chemicals used in this study were analytical grade silver nitrate (AgNO₃ Sigma Aldrich), ciproflaxin, dimethyl sulphoxide (DMSO, Sigma Aldrich), nutrient broth (Himedia), Mueller-Hinton agar (MHA, Merck).

Test microorganisms

Two Gram positive bacteria *Staphylococcus aureus* (ATCC23235), *Streptococcus mutans* (ATCC25175), and one Gram negative bacteria *Klebsiella pneumonia* (ATCC700603) were selected for this study.

Plant collection and preparation of extract

The matured fresh leaves of *Acokanthera schimperi* were collected from Adwa town in Tigray region Ethiopia and authenticated at national Herbarium of Ethiopia, Addis Ababa University (voucher number BT 001). One hundred grams (100 g) of *Acokanthera schimperi* powdered leaves were extracted using a Soxhlet apparatus with distilled water. The extracted material was

then filtered using Whatman No. 1 filter paper and concentrated using rotary vacuum evaporator. Finally, the crude plant extract was taken in an air-tight bottle and stored in refrigerator at 4 °C for further use [15].

Qualitative phytochemical screening

Phytochemical analysis was carried out to identify the secondary metabolites presented in the aqueous crude leaf extracts of *Acokanthera schimperi* according to the standard procedures [16].

Green synthesis of silver nanoparticles (AS-AgNPs)

A solution of 1 mM silver nitrate was prepared by dissolving 0.017 g of silver nitrate in 100 mL volumetric flask using deionized water. Afterwards 10 mL of the prepared aqueous extract was added drop wise into the silver nitrate solution and heated at 80-90 °C for 2 h while stirring [17]. The change in color of the reaction mixture from light brown to brown color indicates the formation of phytogenic silver nanoparticles (AS-AgNPs). The solution mixture was allowed for 24 h at room temperature to complete bio reduction and stabilization of nanoparticles and the solution was centrifuged at 10000 rpm for 20 min. After that the precipitate (AS-AgNPs) was transferred to Petri dish and allowed overnight in the oven to dry and crash using mortal and pistil to make nano powder.

Characterization

The green synthesized AgNPs were primarily confirmed by visual observation, and further characterized by UV-Vis (Shimadzu UV-Vis 2450), FT-IR (Shimadzu FTIR Prestige 21 in the 4000–400 cm^{-1}), XRD (PAN analytical: XPERT-PRO), DLS (Zetasizer Nano ZS90), SEM-EDX (Prisma E SEM) and TEM (Olympus Optical Microscopy) [18].

Standardization of inoculum

The 0.5 McFarland turbidity standard was prepared by adding 0.5 mL of a 1.175% (wt/vol) barium chloride dihydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution to 99.5 mL of 1% (Vol/Vol) sulfuric acid (H_2SO_4). This mixture was considered to be equivalent to the cell density of 1×10^8 CFU/mL. Barium sulphate turbidity was compared with identical tubes containing inoculum 0.85% NaCl saline solution. The turbidity standard is then liquated into test tubes which are identical to those used to prepare the inoculums suspension [19].

Antibacterial activity of AS-AgNPs

To determine *in vitro* antibacterial activities of AS-AgNPs against the selected multidrug resistant, two Gram positive bacteria *S. aureus* (ATCC23235) and *S. mutans* (ATCC25175), and one Gram negative bacteria *K. pneumonia* (ATCC700603), agar a well diffused method was done by measuring zone of inhibition, according to Mazumder *et al.*, [20]. Fifty (50) μL of selected bacterial strains inoculums (1×10^8 CFU/mL) were evenly spread on the surface of solidified Mueller-Hinton Agar (MHA), with sterile cotton swab. The five agar wells of 6 mm diameter were made by using sterile cork borer. 25, 50 and 100 mg/mL concentrations of AS-AgNPs were prepared by using 10% dimethyl sulphoxide (DMSO) solvent and ciproflaxin (20 μg) were dispensed in separate well with the help of a micropipette then plates were kept at room temperature for 1 h to allow uniform diffusion of the AS-AgNPs and ciproflaxin into agar and then incubated at 37 °C for 24 h and the zone of inhibition was measured.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of AS-AgNPs against tested bacterial strains was determined by broth micro dilution method according to Bhattacharyya *et al.*, [21]. The AS-AgNPs were dissolved in DMSO and was prepared with varying concentrations 100, 50, 25, 12.5, and 6.25 mg/mL and ciproflaxin was used as a positive control and DMSO as a negative control. The standardized cultures of bacterial strains were inoculated into 96-well plate microtiter plates along with 100, 50, 25, 12.5, and 6.25 mg/mL of the AS-AgNPs, ciproflaxin (20 µg/mL) and negative control. After incubation at 37 °C for 24 h, the MIC values were determined as the lowest concentration of AS-AgNPs inhibiting visible growth or decreasing turbidity of bacterial strains.

RESULTS AND DISCUSSION

Phytochemical analysis

As shown in Table 1, the current results revealed that aqueous leaf extracts of *Acokanthera schimperi* has shown the presence of alkaloid, phenol compounds, tannins, flavonoid glycoside and steroid which are responsible for phyto-reduction of silver from Ag^+ to Ag^0 , and act as stabilizing, capping agent and prevent the agglomeration and leads to production of nano sized spherical AS-AgNPs. Our results agreed with the previous studies of Chaithanya *et al.* [22], reported that ethanolic leaf extracts of *Acokanthera schimperi* showed the presence of alkaloids, flavonoids, tannins and phenolic compounds and terpenoids.

Table 1. The qualitative phytochemical screening.

Chemical constituents	Test	Result
Test for alkaloid	Mayer's test	++
Phenolic compound	Ferric chloride test	++
Test for tannins	Ferric chloride test	++
Test for flavonoids glycoside	Alkaline reagent test	+
Test for steroid	Liebermann–Buchard's test	+

Characterization of AS-AgNPs

The phyto-synthesis of AS-AgNPs from the aqueous leaf extract of *Acokanthera schimperi* was first observed through a visible color change in the reaction mixture, shifting from light brown to dark brown over three hours. This biotransformation of Ag^+ to Ag^0 from AgNO_3 was accompanied by a gradual intensification of color in the reaction mixture, continuing through a 24 h incubation period. The aqueous leaf extract effectively converted Ag^+ into Ag^0 , with the reduction confirmed by the color change from brown to dark brown. Similar color changes have been reported in earlier studies [22]. These findings align with reports from Velmurugan *et al.* [23], who synthesized silver nanoparticles from southern *Eucalyptus globulus* and observed similar color changes during the bioreduction of Ag^+ to Ag^0 nanoparticles. Additionally, Padalia *et al.* [24], synthesized silver nanoparticles from marigold flowers and also noted the same color transitions.

The UV–Vis spectra obtained from the leaf extract of *Acokanthera schimperi* are presented in Figure 1, covering the range of 300–700 nm. The AS-AgNPs exhibited a prominent absorption peak at 420 nm, which indicates the presence of localized surface plasmon resonance (SPR) due to the oscillation of silver particles, confirming the formation of silver nanoparticles. Earlier studies have noted that SPR silver bands typically appear between 400–480 nm [17]. For

instance, Ghasemi *et al.* [13] reported an absorbance peak at 456 nm for AgNPs synthesized from the aqueous leaf extract of *Rubus discolor*. Similarly, *Berihu et al.* [25] found that TB-Ag NPs from the stem bark extract of *Terminalia brownie* showed an absorbance peak at 428 nm.

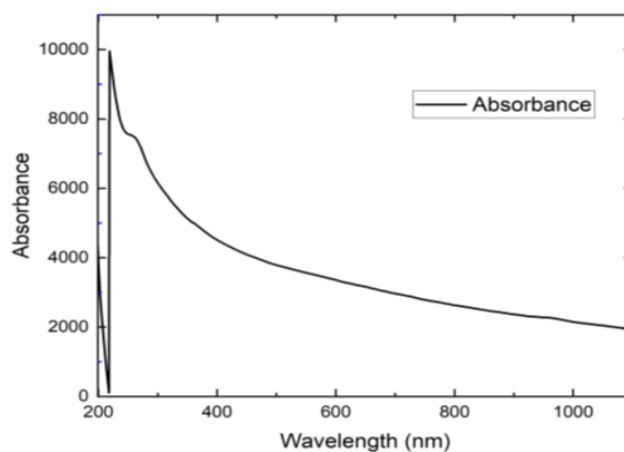


Figure 1. UV-Vis spectrum of AS-AgNPs.

The AgNPs surface chemical composition and stabilizing was determined and characterized using FT-IR spectroscopy. The FT-IR spectrum of *Acokanthera schimperi* leaf extract (Figure 2) (before reaction without AgNO_3) reflected intense peaks at 3752 cm^{-1} (due to phenolic OH stretching), 3339 cm^{-1} (due to $-\text{OH}$ stretching), 1634 cm^{-1} (amide bond of proteins due to carbonyl stretch in proteins), 1077 and 1266 cm^{-1} (due to C-N stretching of aromatic and aliphatic amines) and 561 cm^{-1} (due to C-H of alkynes).

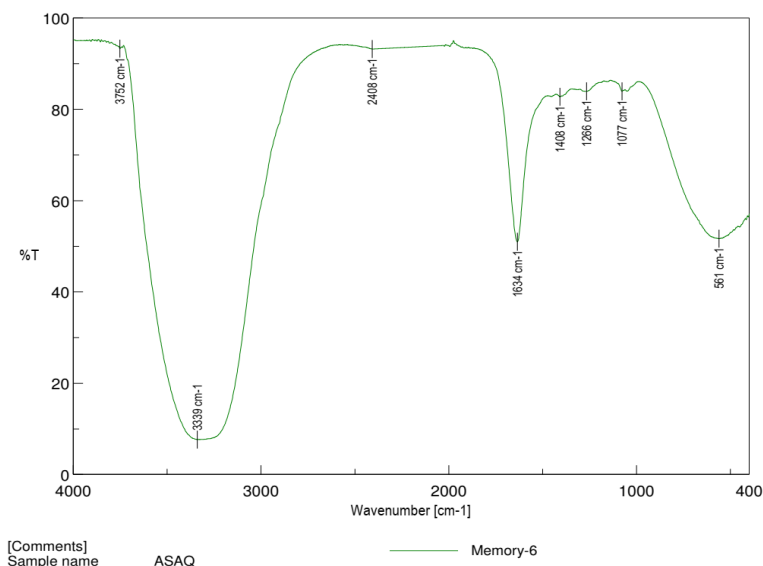


Figure 2. FT-IR spectrums of *Acokanthera schimperi* leaf aqueous extract.

The FTIR spectrum of *Acokanthera schimperi* leaf extracts (Figure 3) (after reaction with AgNO_3) reflected intense peaks at 3714 , 3033 and 2941 cm^{-1} corresponds to amide stretching, O–H alcohol stretching and C–H stretching vibration of methyl or methylene groups. 1739 , 1366 , 1216 cm^{-1} could be due to stretching vibration of carbonyl group of flavonoids, –C–C aromatic groups and ester bonds of phenolic compounds, the final peak at 526 cm^{-1} corresponds to C–H bending. The peaks indicate the reduction of corresponding functional groups. The obtained results suggested that organic compounds in the *Acokanthera schimperi* leaf extract showed the involvement of amides, carbonyl groups and poly phenols in the synthesized AS-AgNPs which might be involved in its formation by acting as capping and stabilizing agents [26]. Indeed, the presence of different functional groups may have contributed to the bio-reduction of Ag^+ ions. Similar observations were noticed in the green synthesis of AgNPs using plant extracts [27, 28].

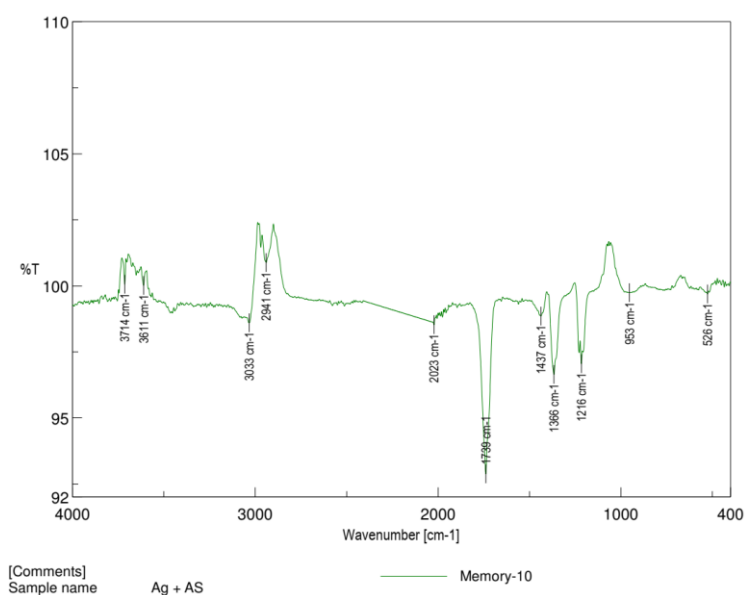


Figure 3. FT–IR Spectrum of the AS-Ag NPs.

The XRD pattern was conducted to confirm the crystalline nature and purity of synthesized nanoparticles (Figure 4). It showed a series of diffraction peaks at 2θ of 32.06 , 37.96 , 44.14 , 64.3 and 77.26 , which corresponding to (101), (111), (200), (220) and (311) planes of AS-Ag respectively, aligning with established pure crystalline silver structures. The sharpness of these peaks indicates the formation of silver structure AS- AgNPs, while the diffraction peaks (111), (200), (220) and (311) represent face-centered cubic silver [29]. The average crystallite size (D) of AgNPs was calculated using Scherrer formula, $D = 0.9\lambda/\beta \cos \theta$, where λ is the wavelength of X-ray radiation, β is the full width at half maximum (FWHM) of the peaks at the diffracting angle θ . The synthesized AgNPs' average crystallite size, as determined by Scherrer's equation, is 53 nm . These results align with previous reports of [30]. Indeed, the presence of picks on planes lower than 30 are due to the existence of phytochemical compounds in the leaf extracts probably, the similar result was reported on the biosynthesis silver nanoparticles [31–33].

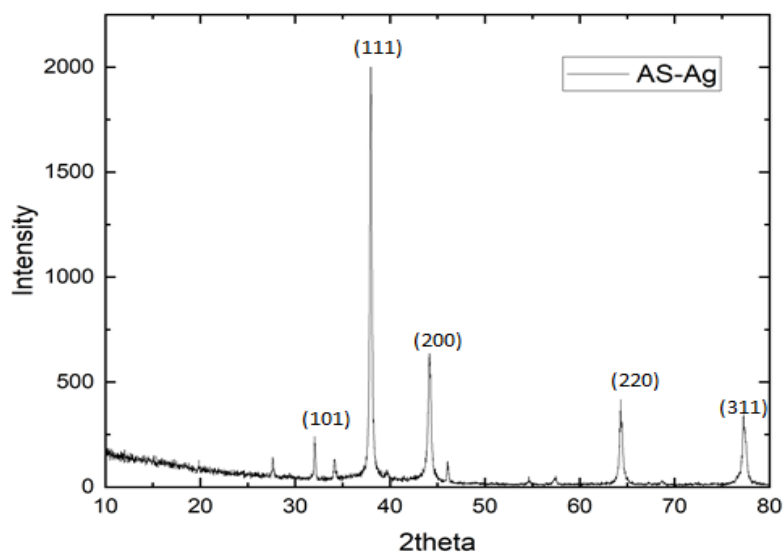


Figure 4. XRD pattern of AS-AgNPs.

The size and morphology of the AS-AgNPs were studied by using TEM, SEM and EDX (Figure 5A-C) techniques. TEM image of AgNPs from the extracted *Acokanthera schimperi* leaf showed that the particles are spherical in shape within the range of 13.5 to 32.8 nm; the average size of the nanoparticles was found to be 20.4 nm (Figure 5A). The size of these biosynthesized Ag nanoparticles is in conformity with the size of earlier reported biosynthesized Ag nanoparticles [34-36]. SEM image of AgNPs synthesized from *Acokanthera schimperi* leaf extract had a particle size of 128.2 nm with majority of the Ag nanoparticles are spherical in shape, with a few agglomerated Ag nanoparticles observed in some places and the extracted leaf is utilized as a reducing and capping agent (Figure 5B). This could be owing to the variable quantities and types of capping agents present in the leaf extract. This is also agreed by the shifts and differences in peak areas seen during the FTIR investigation. The size of these biosynthesized Ag nanoparticles is in conformity with the size of earlier reported biosynthesized Ag nanoparticles [37]. EDX studies were carried out to examine the chemical composition and formation of the prepared sample in addition to verifying the existence of silver nanoparticles. The EDX spectrum of AS-AgNPs showed high intense silver peak at 3.2 keV, which is the characteristic absorption of metallic silver (Figure 5C). Similar reports were observed in *Terminalia arjuna* bark extract [38].

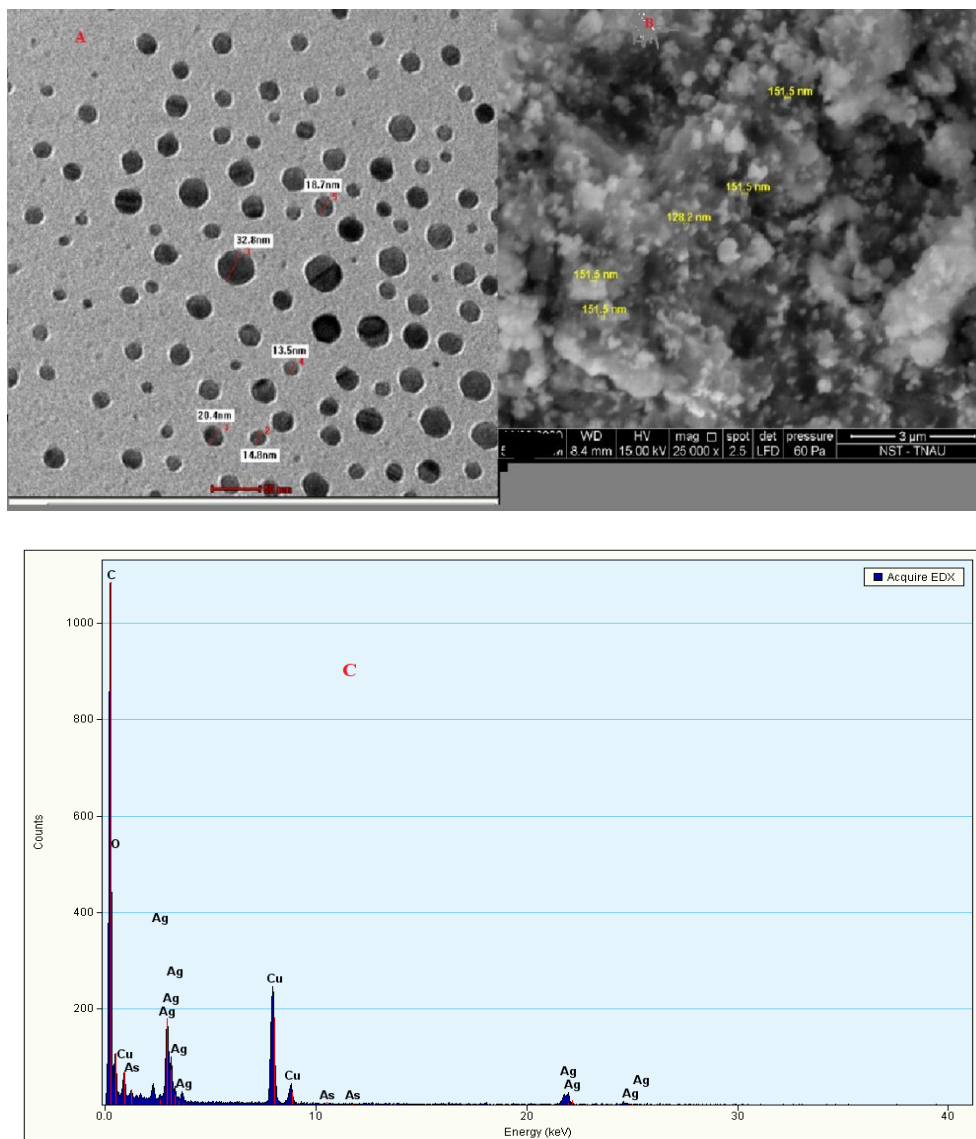


Figure 5. TEM, SEM and EDX images of AS-AgNPs.

The DLS method was used to determine the particle size distribution of the synthesized AS-AgNPs in an aqueous colloidal solution. Using dynamic light scattering (DLS) technique, the size and surface charge of nanoparticles were determined. It was seen that their size varied from 129.5 nm to 415.8 nm with most particles averaging a grain size of 129.5 nm with polydispersity index of 0.435 was displayed on the spectrum (Figure 6) which means that the AS-AgNPs contain particles of various shapes [39]. The DLS measurement was carried out in a fluid phase whereas TEM image was taken in a dry state. Therefore, the particle size in the DLS analysis is larger than in the TEM test. The DLS shows the hydrodynamic diameter of the biomolecules surrounding AgNPs and the intensity-weighted average particle size. Thus, the results of DLS

and TEM cannot be in line with each other, and the results of DLS showed a normally larger size than those of TEM [13].

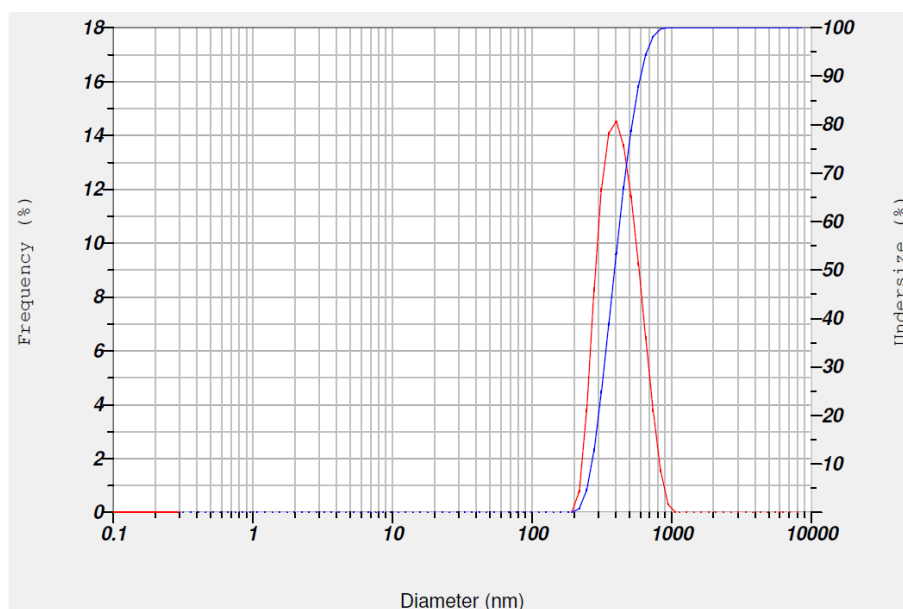


Figure 6. DLS result of the bio-synthesized AS-AgNPs.

Anti-bacterial activity

The inhibitory effect of *Acokanthera schimperi* leaf aqueous extract (AS-AQ) and its green synthesized AS-Ag NPs at range of 25, 50 and 100 $\mu\text{g/mL}$ have been investigated against two gram positive *S. aureus*, *S. mutans*, and one gram negative *K. pneumoniae* bacterial strains by measuring their zone of inhibition in diameters in mm.

Table 2. The zones of inhibition of AS-AQ and AS- AgNPs against multidrug resistant bacteria by agar well diffusion assay (mm).

Strain	Zone of inhibition								DMSO
	AS-AQ $\mu\text{g/mL}$				AS- AgNP $\mu\text{g/mL}$				
	25	50	100	Ciproflaxin (20 $\mu\text{g/mL}$)	25	50	100	Ciproflaxin (20 $\mu\text{g/mL}$)	
<i>S. aureus</i>	-	-	-	15	-	-	-	14	
<i>S. mutans</i>	-	9.1	10.7	32	-	12.3	17.4	32	
<i>K. pneumoniae</i>	-	-	-	41	-	-	8	41	



Figure 7. The antibacterial activity of the AS-AQ and AS-AgNPs (25, 50 and 100 µg/mL) and standard antibiotic ciprofloxacin against three selected bacterial strains.

The results of our investigation displayed in Table 2 shows that, AS-AgNPs has a notable zone of inhibition against tested Gram-positive bacteria *S. mutans* (12.3-17.4 mm) at concentrations ranging from 50-100 µg/mL, compared with AS-AQ extract (Figure 7). In addition, the AS-AQ extract showed moderated antibacterial activity against tested Gram-positive bacteria *S. mutans* (9.1-10.7 mm) at concentrations ranging from 50-100 µg/mL. As was seen in Figure 7, AS-AgNPs have shown efficient zone of inhibition against *S. mutans* and *K. pneumoniae* compared with AS-AQ extract. These findings are in agreement with Alburae *et al.* [40], study reported that green synthesized silver nanoparticles from using *Lasiurus scindicus* and *Panicum turgidum* seed extracts have shown substantially zone of inhibition against *S. mutans* compared to their respective seed extract. Similarly, these findings are consistent the report of Mahal *et al.* [41], which demonstrated that green-synthesized copper nanoparticles from *Piper longum* fruit extract showed a notably higher zone of inhibition against *K. pneumoniae* than the crude seed extract. Raghupathy *et al.* [42], reported that silver nanoparticles which was prepared from aqueous leaf extract of *Psidium guajava* had shown strong antibacterial activity against tested bacterial strains against *S. aureus*, *S. mutans* and *E. faecalis*.

In comparison, antibacterial analysis revealed that AS-AQ mediated green synthesized AS-AgNPs have shown enhanced broad spectrum antibacterial activity against tested bacteria due to the bioactive compounds present, in AS-AQ acting as capping agent and also enhances the bacterial cell up taking capacity and in addition their tunable physico and chemical properties of bioactive corona on surface of AS-AgNPs depicted substantial antibacterial activity [43, 44].

CONCLUSION

In the present study, aqueous leaf extract *Acokanthera schimperi* was used as green synthesis of bio inspired effective as antibacterial agent. The green synthesized *Acokanthera schimperi* of AgNPs was confirmed by UV-Vis spectroscopy analysis and the XRD pattern of the produced nanoparticles revealed their crystalline nature, and the size of AgNPs was reported to be 53 nm

by XRD analysis. Indeed, the synthesized AS-AgNPs was analyzed by TEM images and it reveals that in spherical shaped, with an average size of 20.4 nm. The studied AS-AgNPs demonstrated good antibacterial effect against *S. mutans* and *K. pneumoniae*, so it can be used in biomedical applications. However, further research is recommended to conduct additional studies in order to support the molecular mechanism of therapeutic action of AS-AgNPs against tested bacterial strains.

Compliance with ethical standards

This study does not involve any human or animal subjects.

Declaration of competing interest

The authors declare that no conflict of interest

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