GREEN SYNTHESIS OF SILVER NANOPARTICLES OF *TAGETES MINUTA* L. LEAVES AND ITS POTENTIAL ANTIBACTERIAL ACTIVITY

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(Received 20th Dec 2023; accepted 25th Mar 2024)

Abstract. The plant mediated synthesis of metallic nanoparticles is a fast-growing area of interest in the field of nanotechnology due to its eco-friendly approach. This study demonstrated the green synthesis of silver nanoparticles (AgNPs) using *Tagetes minuta* leaf extract. The AgNPs were characterised using UV-visible spectroscopy, transmission electron microscopy (TEM), energy dispersive X-ray (EDX) analysis and Fourier transform infrared (FTIR) spectroscopy. UV–visible spectrum of synthesised silver nanoparticles showed maximum peak at 442 nm. TEM revealed that the particles were spherical in shape and size ranging from 10 to 50 nm. The EDX spectrum confirmed the presence of silver metal. Preliminary antibacterial activity was determined using the agar well diffusion method, which showed growth inhibition against selected gram-positive and gram-negative bacteria.
Keywords: *Ag^+ ions, energy dispersive X-ray, FTIR spectroscopy, plant extract, TEM, UV-visible spectrometry*

Introduction

Nanotechnology is the blanket term for the synthesis, manipulation, and application of materials and structures that range between 1 and 100 nm and is currently one of the most active research fields in material science (Sarsar et al., 2013; Padalia et al., 2015; Khatoon et al., 2017; Alabdallah et al., 2021; Oves et al., 2022). Size, distribution, and morphology of nanoparticles determines their biological effectiveness (Krishnamurthy et al., 2012; Sivakumar, 2021; Mustapha et al., 2022). Noble metal nanoparticles such as gold, silver, and platinum show promising applications in the fields of biotechnology, bioengineering, solar energy conversion, medicine, and water treatment (Dahl et al., 2007; Sharma et al., 2009; Sivakumar, 2021). Traditionally, nanoparticles are produced using chemical and physical methods, but these methods often face several caveats in the way of costs and environmental damage (Ramya and Subapriya, 2012; Velidandi et al., 2020; Islam et al., 2021). Green chemistry and green synthesis methods have since been adopted in the efforts of reducing both costs and generated hazardous waste, as well as decreasing the risk of safety concern over the products (Kaler et al., 2010; Velidandi et al., 2020). The biological method of synthesizing nanoparticles occurs by redox reaction that builds towards larger and more complex systems beginning at the molecular level (Ramya and Subapriya, 2012). Based on green chemistry perspectives, the selection of an

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DOI: http://dx.doi.org/10.15666/aeer/2203_22112224

environmentally benign solvent and nontoxic chemicals in the synthesis of nanoparticles are imperative (Sharma et al., 2009; Tiwary and Jha, 2017). Natural material from microorganisms, enzymes, and plants are used for green synthesis of silver nanoparticles (Kotakadi et al., 2014; Vidhu and Philip, 2014; Oves et al., 2022).

Silver has been used as an antimicrobial agent for many decades, but the rising interest in the properties of metallic silver in the form of silver nanoparticles (AgNPs) is focused towards the increasing threat of antibiotic resistance (Roy and Das, 2015; Ojo et al., 2018). Ahmed et al. (2016) claimed that silver is the least toxic metal to animal cells that is used as an antimicrobial and is effective against over 650 microorganisms from different classes such as gram-negative and gram-positive bacteria, fungi, and viruses. In order for silver to have any antimicrobial properties, it must first be in its ionized form, as the positive charge on the Ag^+ ions is vital in forming nanoparticles (Ahmed et al., 2016; AlSalhi et al., 2016). In conjunction, the medicinal properties of plants are attributed to its phytochemicals, which are in a position to reduce, cap, and stabilise Ag^+ ions (Chinnasamy et al., 2017; Tiwary and Jha, 2017). Organic chemicals present in plant aqueous extracts (e.g. carbohydrates, proteins, phenols, flavonoids, terpenoids, alkaloids) are capable of donating an electron that results in the reduction of Ag^+ ions to elemental Ag (Roy and Das, 2015; Tiwary and Jha, 2017). Nanotechnology also allows for the exploitation of antimicrobial properties of silver, as they are used in the form of nanoparticles. AgNPs display a large surface area to volume ratio – which allows for broad contact with the nuclear content of the bacteria, thus enabling the inactivation of DNA replication leading to growth inhibition (Ojo et al., 2017). It has been reported that AgNPs attach to the cell walls of bacteria and disturb the cell wall permeability and cellular respiration (Singh et al., 2008; Roy and Das, 2015).

Tagetes minuta is an aromatic herbaceous plant belonging to the Asteraceae family that has a high-grade essential oil with numerous uses in beverage, cosmetic, and pharmaceutical industries (Shirazi et al., 2014; Martinez et al., 2020; Rikisahedew et al., 2023). It is found along riverbanks, forest margins, dry wooded valleys and hillsides in KwaZulu-Natal since its naturalisation in South Africa. The leaves of *T. minuta* are used in the preparation of traditional remedies for the management of stomach ailments, headaches, diarrhoea, malaria, and epilepsy (Karimian et al., 2014; Kyarimpa et al., 2014; Igwaran et al., 2017). The pharmacological effects of the leaf extracts are purported to be the result of various secondary metabolites including essential oils particularly abundant in monoterpenes, sesquiterpenes, flavonoids, aromatics, and thiophenes (Bansal et al., 1999; Brene et al., 2009; Sadia et al., 2013; Rikisahedew et al., 2023). The aims of this study were to employ the use of aqueous leaf extracts of *T. minuta* in the biosynthesis of AgNPs, characterise the particles, as well as to observe its antimicrobial activity against both gram-positive and gram-negative bacteria.

Materials and Methods

Collection of plant material

Fresh leaves of *T. minuta* were collected from the University of KwaZulu-Natal (UKZN), Westville Campus, located in Durban (29°49'01.0"S 30°56'51.2"E), South Africa. The leaves of *T. minuta* were carefully cleaned using double distilled water and then subjected to air-drying at a temperature of 24°C for a period of 6 weeks. To ensure proper identification and documentation, a voucher specimen was prepared and deposited

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at the UKZN Westville Herbarium, with the accession number 18216 and voucher number 01.

Methanol extraction

For the methanol extraction, the air-dried leaves were finely ground into a powder using a domestic spice mill manufactured by Kenwood Ltd, Havant, UK. A total of 30 grams of the ground material was placed in a round bottom flask, along with 50 ml of methanol of high-performance liquid chromatography (HPLC) grade. The flask was then connected to a Soxhlet apparatus and subjected to boiling for two 3-hour sessions. This process resulted in the production of the plant extract. To remove any impurities, the extract was filtered using Whatman No. 1 filter paper and subsequently stored in a mason jar placed in a dark environment at a temperature of 4°C.

Fresh water extraction

For freshwater extraction, 10 grams of fresh leaves were meticulously washed with distilled water and subsequently ground into a fine powder using the same domestic spice mill as mentioned earlier. The powder was then boiled for 10 minutes in 100 ml of distilled water. The resulting mixture was filtered using Whatman No. 1 filter paper and stored as a fine powder. The filtrate obtained from this process was utilized for the synthesis of silver nanoparticles.

Green synthesis of silver nanoparticles (AgNPs)

The synthesis of AgNPs using a green method was conducted with slight modifications to the procedure described by Sudha et al. (2017). In this method, 1 mL of the methanolic extract was added to a 19 mL solution of 1 mM aqueous silver nitrate ($AgNO_3$). The mixture of $AgNO_3$ and methanolic extract was combined in a conical flask and incubated in a water bath at a temperature of 60°C for a duration of 30 minutes. The formation of AgNPs was indicated by a color change from yellow to brown. The resulting solution was then subjected to centrifugation at a speed of 16,000 rpm for 5 minutes using an Eppendorf Centrifuge 5415R. After removing the supernatant, deionized water (diH_2O) was added to the Eppendorf tube and the centrifugation process was repeated three times. The resulting pellet was suspended in 10 mL of diH_2O, vortexed, and used for further characterization.

Characterisation of AgNPs

a) UV–Visible spectroscopy

The presence of AgNPs was confirmed through UV-vis spectroscopy. The absorbance spectrum was recorded using a UV-1800 Shimadzu spectrophotometer (Tokyo, Japan), with the absorbance wavelength range set at 350-700 nm. A blank solution of diH_2O was used as a reference.

b) Energy dispersive X-ray analysis (EDX)

Energy dispersive X-ray analysis (EDX) was conducted to determine the presence of elemental silver in the synthesized AgNPs. A drop of the synthesized AgNPs was placed on an aluminum stub and left to dry for 24 hours. The stub was then sputter coated in a Polaron SC500 Sputtercoater. EDX microanalysis was performed using an Ultra Plus

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Field Emission Gun Scanning Electron microscope (FEGSEM) (Carl Zeiss, Munich, Germany) in conjunction with AzTec Analysis Software (Oxford Instruments, High Wycombe, UK) to determine the presence of elemental silver in the sample.

c) Fourier transform infrared (FTIR) spectral analysis

The identification of the capping and reducing agents was achieved through FTIR analysis. In order to prepare the samples, centrifugation was performed at a speed of 10000 rpm for a duration of 30 minutes using the Beckman Coulter Avanti J-E Centrifuge. The resulting pellet was then suspended in deionized water and utilized for further characterization. Spectroscopic measurements of the samples, with a volume of 200 µl, were conducted using a Perkin Elmer FTIR Spectrum 100 spectrophotometer (MA, USA) at a wavelength range of 350-700 nm.

d) High resolution transmission electron microscopy (HRTEM)

HRTEM was employed to examine the overall micromorphology, including the shape and size, of the synthesized AgNPs. A drop of sonicated AgNPs was placed on a copper grid and allowed to dry for a period of 20 minutes. The HRTEM JEOL 2100 instrument was used for observation, and the particle size was determined using iTEM software (Soft Imaging System GmBH, Münster, Germany).

Antibacterial screening

The antibacterial activity of the synthesized AgNPs was accomplished using the agar well diffusion method, as described by Saif et al. (2017), against various bacterial strains including *Escherichia coli* (ATCC 25218), *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (ATCC BAA-1683), *Bacillus subtilis*, and *Pseudomonas aeruginosa* (ATCC 25215). Prior to the screening, the bacterial strains were cultured for 18 hours in Nutrient Broth (Biolab, Midrand, South Africa) at a temperature of 37°C with agitation. The turbidity of the bacterial samples was adjusted by suspending them in sterile distilled water (dH_2O) using a 0.5 McFarland standard. The medium for the agar well diffusion assay was prepared by dissolving 38 g of Mueller Hinton agar (MHA) in 1 L of sterile distilled water. The medium was then subjected to boiling for 1 minute and autoclaving at 121°C for 20 minutes. After cooling to room temperature (24°C), the medium was poured into sterile petri dishes and allowed to solidify under a sterile environment. Each bacterial strain was evenly spread onto the MHA plates and left to dry. Wells with a diameter of 5 mm were aseptically created using a sterile cork borer. Subsequently, 90 µl of the AgNP stock solution (1 mg mL^{-1}) was pipetted into each well, considering the depth of the well. The petri dishes were then placed in incubators at various temperatures to facilitate the diffusion of the solution through the MHA. The inhibition zones were observed after 18 hours, and all experiments were conducted in triplicate. The zone of inhibition was measured for antibacterial activity and presented as mean values ± standard error.

Statistical analyses

The antibacterial screening was done in triplicate. One-way analysis of variance (ANOVA) and paired Sample t-tests were conducted to compare the effects of methanolic extract and antibiotic treatments on bacterial strains. Descriptive statistics for each condition are presented as mean ± standard deviation. Significance was set at $\alpha = 0.05$.

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All analyses were conducted using R studio software (RStudio Team, 2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL https://www.rstudio.com/) and error bars were shown as standard deviation unless otherwise stated.

Results and Discussion

The fresh suspension of *T. minuta* was a light green in colour (*Figure 1a*). However, after the addition of silver nitrate and exposing to heat in a 60°C water bath for 30 mins, the suspension turned a dark brown colour (*Figure 1b*). The reduction of silver ions into silver nanoparticles during exposure to the plant extract is indicated by this colour change. It appears that the intensity of the colour reaction was directly proportional to the formation of the AgNPs. The UV-vis spectra recorded from the methanolic extract of *T. minuta* is presented in *Fig. 2*, where the maximum absorption peak is shown to be at 442 nm. Surface plasmon resonance (SPR) is the collective oscillation of electrons in the conduction band on a nanoparticle surface (Suman et al., 2014). Siddiqui et al. (2018) claimed that the absorption band from 400 to 500 nm represents the dipole component of the SPR of silver nanoparticles. This implies that the peak wavelength, width, and effect of these resonances yield a unique spectral fingerprint for a plasmonic nanoparticle with a distinct size and shape. Using high-resolution TEM, Mock et al. (2002) showed that silver nanoparticles that peak in the range of 410-500 nm are spherical, whereas in the range of 500-700 nm, particles are usually triangular or pentagonal. This observation strongly suggests that the AgNPs synthesised in this study were spherical in shape.

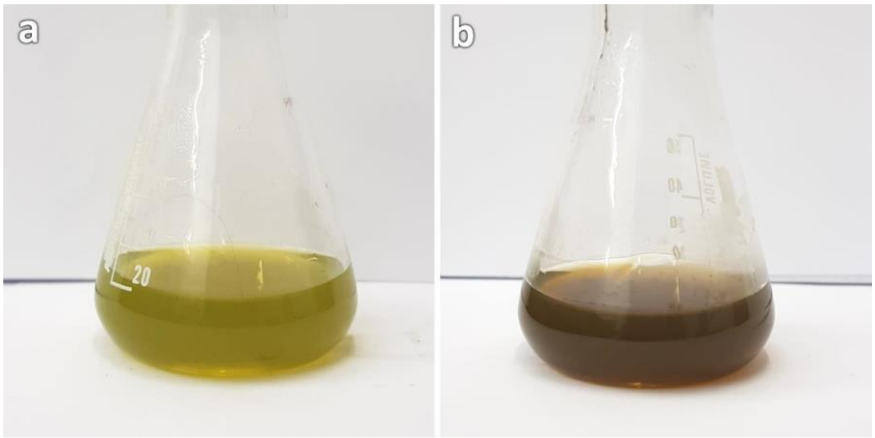

Figure 1. *Images of silver nanoparticle synthesis using T. minuta extract from leaves: a) Silver nitrate solution with leaf extract. b) Synthesized silver nanoparticle solution after heating for 30 minutes*

The shape and size of the AgNPs from *T. minuta* leaf extracts are depicted in *Figure 3*. The optical and electronic properties of AgNPs are significantly influenced by their shape (Kim et al., 2007). The particles appear to be uniformly spherical. The sizes ranged from 7-42 nm and the average diameter was found to be 11.75 nm (*Fig. 4*). Similarly-shaped silver nanoparticles were synthesised using *T. patula* leaf extract (Elemike et al., 2018) and *T. erecta* flower extract (Padalia et al., 2015). The AgNPs were predominantly monodispersed and stable. Organic material derived from the plant extraction process

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causes the inherent functional group capping, which in turn offers stability and prevents agglomeration (Shaik et al., 2014).

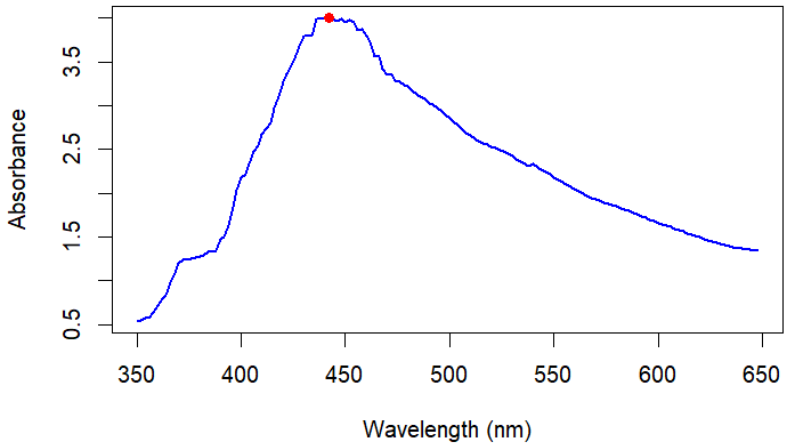

Figure 2. *UV-Vis absorption spectra of reduction of silver ions to silver nanoparticles after 30 min reaction, peaking at 442 nm*

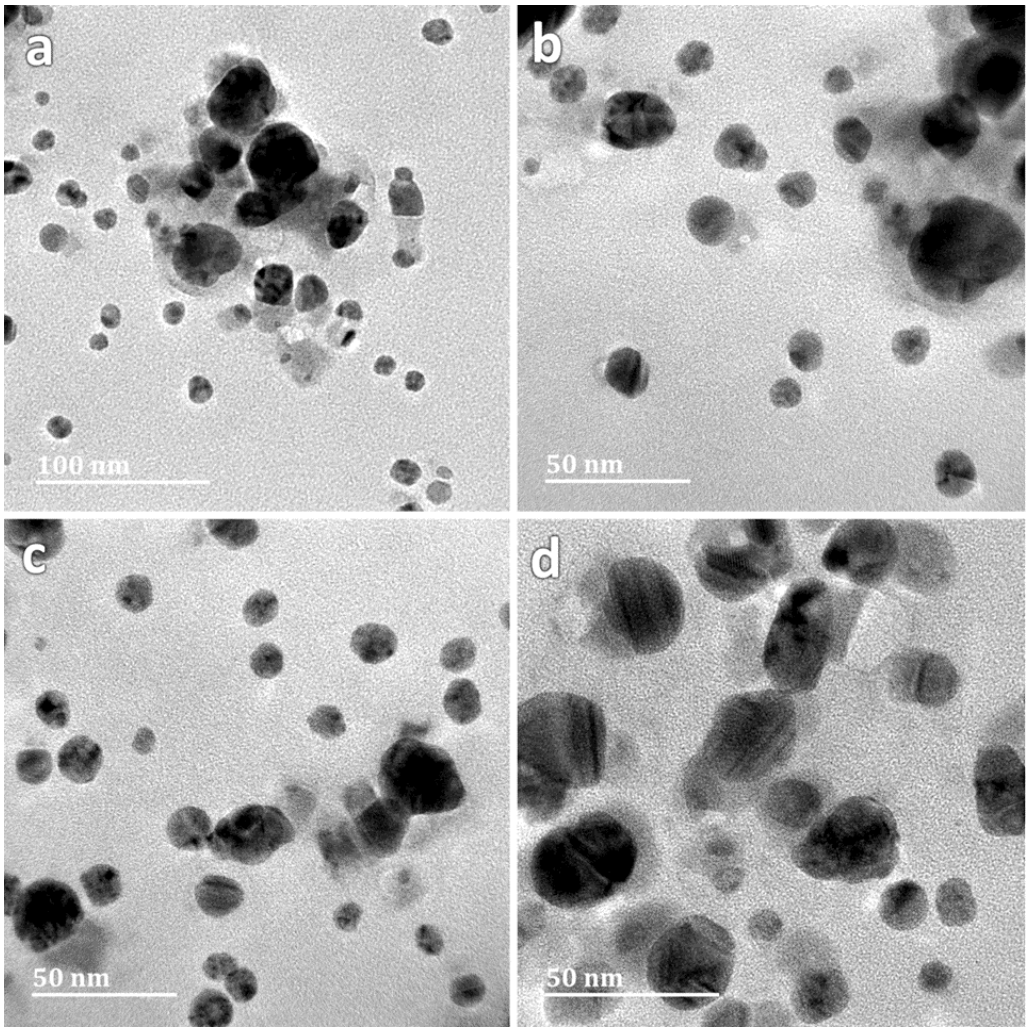

Figure 3. *HR-TEM images of silver nanoparticles at low and high magnification*

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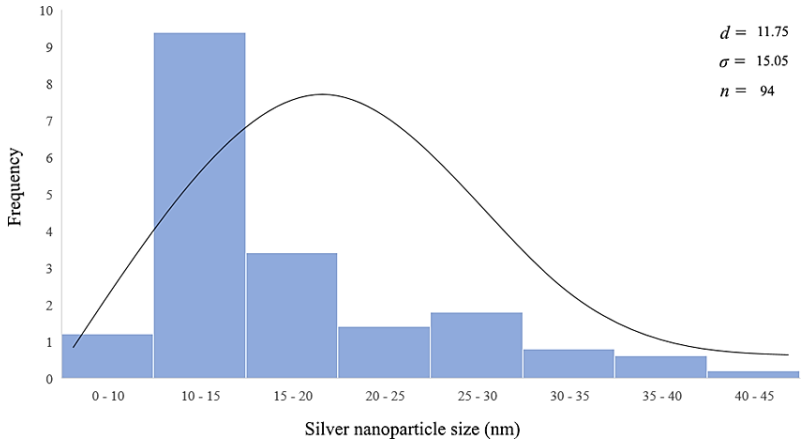
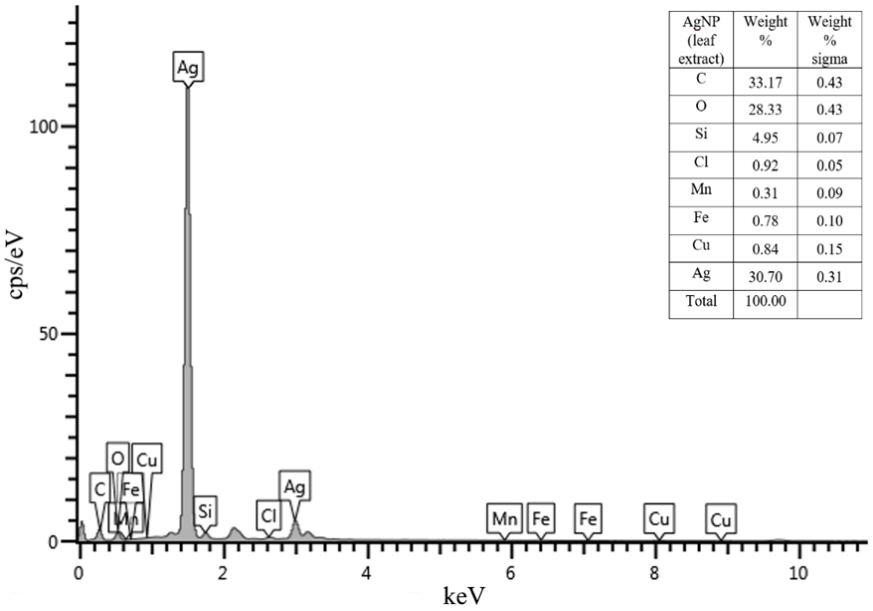


Figure 4. *Frequency histogram for silver nanoparticle size range*

A strong signal of silver is evident from the EDX spectrum at 1.5 keV and a weaker signal at 3 keV (*Figure 5*) confirming the presence of elemental silver. A similar spectral profile for silver has been reported by Hedaginal and Taranath (2017) for the leaf extract of *Thunbergia alata*. Weak signals of carbon, oxygen, iron, manganese, silicon, chlorine, and copper are also evident. The presence of silicon, carbon, and copper is likely to be from the support glass cover slip and grid used in the methodology for EDX. Signals of other trace elements may be the result of x-ray emission from organic compounds involved in the capping of the nanoparticles and are bound to the surface of the nano-silver (Singh et al., 2015).



AgNP (leaf extract)	Weight %	Weight % sigma
C	33.17	0.43
O	28.33	0.43
Si	4.95	0.07
Cl	0.92	0.05
Mn	0.31	0.09
Fe	0.78	0.10
Cu	0.84	0.15
Ag	30.70	0.31
Total	100.00	

Figure 5. *EDX spectrum of synthesised silver nanoparticles using leaf extract of Tagetes minuta*

The known active ingredients in *T. minuta* are terpenoids viz. limonene, caryophyllene, and eucarvone (Tiwari et al., 2016; Igwaran et al., 2017); amines viz. pyrrolidine (Meshkalasadat et al., 2010); ketones viz. tagetone, dihydrotagetenone,

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tagetenone (Gil et al., 2002; Mohammad et al., 2010); and hydrocarbons viz. ocimene (Igwaran et al., 2017; Rikisahedew et al., 2023). The FTIR spectrum analysis of biosynthesised silver nanoparticles are displayed in *Fig. 6* which manifest absorption peaks located at the regions between 600 cm^{-1} and 3300 cm^{-1} in order to identify the functional groups of the extract involved in the reduction of the synthesised AgNPs. Prominent peaks on the FTIR spectrum are marked at 3272 cm^{-1}, 2119 cm^{-1}, 1637 cm^{-1}, 1016 cm^{-1}, and 595 cm^{-1}. The absorption peak at 3272 cm^{-1} is assigned to –OH stretching in alcohols and phenolic compounds, which indicates the possible involvement of the known terpenoids (Awwad et al., 2013; Elemike et al., 2018). The peak at 2119 cm^{-1} suggest –C≡C and –CH groups that are found in terpenoid ring structures and is close to that reported for aliphatic aldehydes (Shaik et al., 2014; Hedaginal and Taranath, 2017). The absorption peak at 1637 cm^{-1} arose due to C=C stretches that are typical of aliphatic and aromatic amine structures and alkenes (Jha et al., 2018). Peaks below the value 1300 cm^{-1} are usually indicative of C–C and C–O groups but are not as reliably interpreted due to a larger number of different vibrations (Kumar et al., 2017).

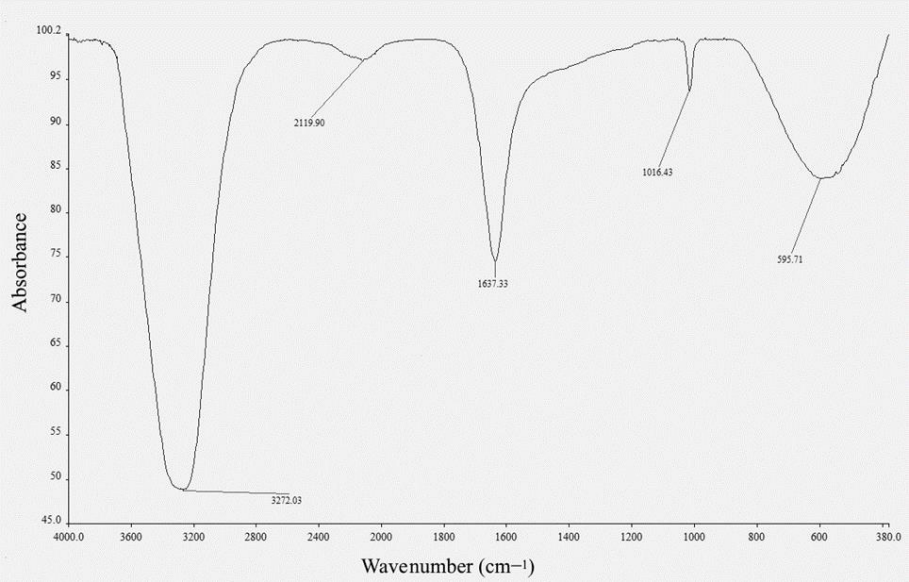

Figure 6. *FTIR spectrum of the synthesised silver nanoparticles using leaf extract of Tagetes minuta*

The antibacterial efficacy of silver nanoparticles synthesized from *T. minuta* leaves are summarized in *Table 1* and were assessed by measuring the diameter of the zone of inhibition of each well. The AgNPs exhibited varying degrees of inhibition against 5 bacterial strains (MRSA, *E. coli, S. aureus, B. subtilis*, and *P. aeruginosa*). The antibiotic used for the gram-positive bacteria was streptomycin and gentamycin for gram-negative bacteria. In general, the silver nanoparticles were more effective against gram-positive than gram-negative bacteria. The AgNPs were least effective against *E. coli* and *P. aeruginosa*, which showed inhibition zones very similar to that of the antibiotic (gentamycin). Sondi and Salopek-Sondi (2014) demonstrated silver nanoparticles penetrating the bacterial cell walls of *E. coli*, but growth inhibition was dependent on the concentration of the AgNPs used, and in most instances would only delay the growth of the bacterial colonies. Pal et al. (2007) studied the effects of nanoparticle shape against

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gram-negative bacteria and found that spherical and rod-shaped nanoparticles are less effective than triangular-shaped nanoparticles. This may explain the ineffectiveness of the AgNPs synthesised in this study against *P. aeruginosa*. Despite the methanolic extract showing a stronger antibacterial activity against these 5 bacteria (*Figure 7*), the experimental design also shows the ability to produce AgNPs from the leaves of *T. minuta*.

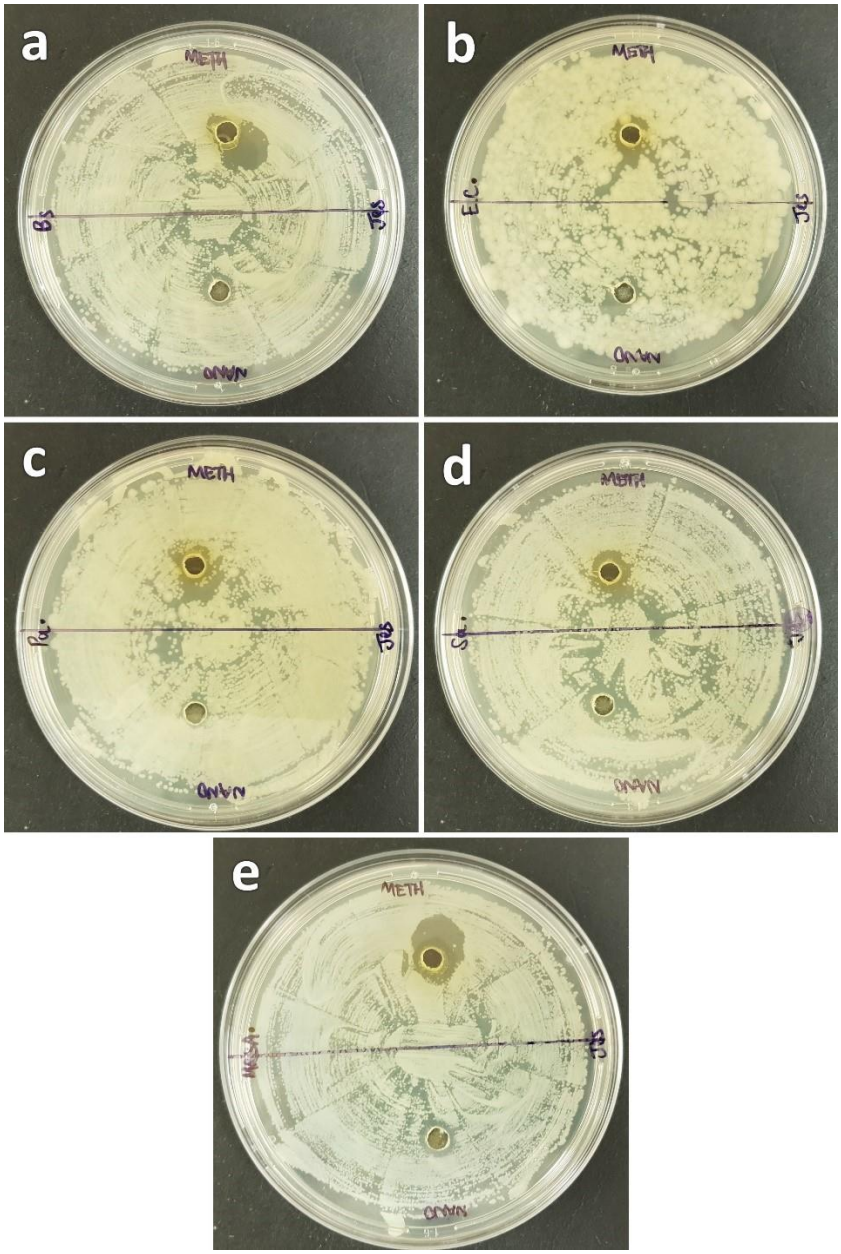

Figure 7. *Antibacterial activity of methanolic leaf extract and AgNPs synthesised from the leaves of T. minuta against gram positive and gram-negative bacteria: a) Bacillus subtilis. b) Escherichia coli. c) Pseudomonas aeruginosa. d) Staphylococcus aureus. e) Methicillin-resistant Staphylococcus aureus*

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Table 1. *Antibacterial activity of T. minuta leaves extract*

Bacterial Strain	Treatment	Mean	Standard deviation	t(df)	95% Confidence Interval	Mean difference
MRSA	Methanolic extract	15.66667	2.08167	12.758	[10.16219, 20.50448]	15.33333
	Antibiotic	0.333333	0.471405			
E.coli	Methanolic extract	10.33333	1.24722	-3.355	[-16.73813, 2.07146]	-7.333333
	Antibiotic	7.66667	4.04124			
S. aureus	Methanolic extract	10.33333	0.471405	31	[8.899116, 11.767551]	10.33333
	Antibiotic	0	0			
B. subtilis	Methanolic extract	12.33333	1.24722	5.547	[1.495522, 11.837812]	6.666667
	Antibiotic	5.66667	0.471405			
P. aeruginosa	Methanolic extract	12.66667	1.88562	2	[-3.837755, 10.504421]	3.333333
	Antibiotic	9.333333	1.24722			

Data presented are means ± standard error. Significance was set at $\alpha = 0.05$, n=3 for all samples

The bacterial growth of the gram-positive bacteria (MRSA, *S. aureus*, and *B. subtilis*) was more severely inhibited by the AgNPs synthesised in this study. Guzman et al. (2012) highlighted the importance of the size of AgNPs against gram-positive bacteria, concluding that silver particles between 9 and 14 nm in diameter showed the highest activity. In *Figure 4*, it is evident that the silver nanoparticles synthesised in this study that were under 15 nm in diameter were the most frequent, which likely contributed to the higher antibacterial activity. It has been reported that AgNPs induce the inactivation of DNA replication and thus protein synthesis in gram-positive bacteria by interacting with present thiol groups (Ojo et al., 2017).

Conclusions

This study provided a simple and rapid method for the biosynthesis of AgNPs using *T. minuta* aqueous leaves extract. The secondary metabolites present in *T. minuta* perform the dual function of formation and stabilisation of AgNPs through the action of various terpenoid and amine compounds identified. The AgNPs were further confirmed by using UV-Vis spectroscopy, EDX, and FTIR techniques. The shape of the nanoparticles was determined to be spherical using HR-TEM. The biosynthesised AgNPs also exhibited antimicrobial activity against selected gram-positive and gram-negative bacteria strains, which is in part due to the size and shape of the AgNPs. Hence, this study supports the technique of plant mediated green synthesis of AgNPs, which has the potential to be utilised in various clinical applications for other plant species.

Acknowledgements. Funding by the National Research Foundation (NRF) is greatly appreciated. The authors acknowledge Researchers Supporting Project number (RSP-2024R375), King Saud University, Riyadh, Saudi Arabia.

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DOI: http://dx.doi.org/10.15666/aeer/2203_22112224

Data availability statement. The present research is part of the first author's MSc thesis entitled" Histo-phytochemical evaluation and characterisation of the foliar structures of *Tagetes minuta* L. (Asteraceae)"– https://researchspace.ukzn.ac.za/server/api/core/bitstreams/231d147b-c55e-4f05-a226-900d4ef327b5/content

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