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Synthesis of silver nanoparticles using crude leaf extracts of *Acacia nilotica, Azadirachta indica*, *Carissa spinarum, Melia azedarach, Senna didymobotrya* and *Warburgia ugandensis,* and their antifungal activity against *Sporisorium scitamineum*

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Bio-synthesised silver nanoparticles are effective in controlling several micro-organisms. They are correspondingly environmentally friendly, affordable, and easy to synthesise when compared with chemically synthesised silver nanoparticles. This study investigated the efficacy of biosynthesized silver nanoparticles against the fungus *Sporisorium scitamineum*, the causal agent of sugarcane smut. The reduction of silver nitrate upon mixing with the plants' crude extracts was evidenced by the change in colour of the mixture to dark brown. Optimization of the mixtures using ultraviolet-visual spectroscopy showed peaks in the range of 340 to 450 nm. The Fourier transform infrared spectroscopy analysis identified proteins to be essential capping agents, and reducing sugars were responsible for the reduction of silver nitrate to nanoparticles and stabilizing the nanoparticles. The transmission electron microscope analysis showed the sizes of the nanoparticles to vary between 3 and 70 nm. *Carissa spinarum* and *Melia azedarach* had the most antifungal activity against *S. scitamineum* as observed from the inhibition-zone assay. Silver nanoparticles were successfully synthesized using the selected botanicals. All the synthesized nanoparticles showed varying antifungal effects against the *S. scitamineum. C. spinarum* and *M. azedarach* exhibited the highest antifungal activity, while *Azadirachta indica* showed the least.

Key words: *Sporisorium scitamineum*, *Acacia nilotica, Carissa spinarum, Senna didymobotrya, Warburgia ugandensis, Melia azedarach, Azadirachta indica,* bio-synthesised silver nanoparticles, antifungal activity.

INTRODUCTION

Traditionally, silver has been known and used for its antimicrobial activity (Jamiu and Bello, 2018). When reduced to their nano-form, silver nanoparticles (AgNPs) possess novel and more efficient antimicrobial properties, owing to their large surface to volume ratio, size, shape and structure (Rafique et al., 2017).

Metal nanoparticles have been traditionally produced by physio-chemical methods that include ion sputtering or pulsed laser ablation, reduction, solvothermal synthesis, hydrothermal and sol-gel methods. Recently, there have been environmentally friendly synthesis methods that use natural products that have been termed the “green synthesis” or “biosynthesis” of nanoparticles (Chouhan, 2018; Vala et al., 2021).

Chemical and physical methods are generally expensive, harmful and inflammable, unlike the biosynthesis method which is cost-effective, energy-saving and environmentally benign as it uses microorganisms and plant extracts. The phytochemicals such as lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides, and enzymes present in biological material are used as reducing, capping and stabilizing agents. The use of agricultural waste helps in reducing the cost of producing the AgNPs as well as limiting the need of using hazardous chemicals and therefore encourages the “green synthesis” production (Chouhan, 2018; Hemlata et al., 2020). The use of plant extracts has been proven to be affordable, easy to bulk up, simple and environmentally friendly (Sanchooli et al., 2018; Raza et al., 2021).

Biosynthesised silver nanoparticles (b-AgNPs) have been found to have antibacterial, antifungal, and antiviral properties, with no environmental concerns and development of microbial resistance. These characteristics have ignited increasing interest in the synthesis of silver nanoparticles (Velu et al., 2017; Hemlata et al., 2020).

Upon synthesis of the AgNPs are synthesised, it is essential to characterise them to understand their physicochemical properties which could have an impact on their biocompatibility during use. The characterization is aimed at understanding the size, size distribution, shape, surface area, stability and aggregation of the particles (Zhang et al., 2016). Characterizing can be done through various analytical techniques which include ultraviolet-visible (UV-Vis) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, X-ray diffractometry (XRD), dynamic light scattering (DLS), transmission electron microscopy (TEM) and atomic force microscopy (AFM) (Khatoon et al., 2017; Al-zubaidi et al., 2019; Jamiu and Bello, 2018; Khan and Javed, 2021).

Upon characterising the b-AgNPs, they can then be used for various purposes which include using them as an antifungal treatment. Overcoming fungal diseases is difficult, especially because of the limitation of several antifungal remedies as well as the environmental impacts that are caused by chemical treatments (Zhang et al., 2016).

Biosynthesised silver nanoparticles have shown exceptional antifungal activity against several phytopathogenic fungi including *Rhizoctonia solani, Alternaria alternata, Sclerotinia sclerotiorum, Botrytis cinerea, Macrophomina phaseolina* and *Curvularia lunata* at the concentration of 15 mg (Zhang et al., 2016).

Several plants have been found to possess medicinal and antimicrobial properties. These properties are attributed to the presence of phytochemicals such as limonoids, flavonol glycosides, saponins, steroids, terpenes, and tannins (Ibrahim et al., 2010; Jafari et al., 2013; Okello and Kang, 2019). The leaf extracts may possess variable effects on pathogens, but have been found to have a higher efficacy when they are used as b-AgNPs (Chouhan, 2018).

The objective of this study is to develop, characterise, and evaluate AgNPs synthesised from the leaf extracts of known antimicrobial plants against *Sporisorium scitamineum* (Syd) M. Piepenbr., M. Stoll & Oberw., the fungal pathogen that causes sugarcane smut (Bhuiyan et al., 2021). Hitherto, only *Azadirachta indica* and *Melia azedarach*, among the selected botanicals, have been used to synthesize AgNPs, yet their antifungal efficacy has not been evaluated against *S. scitamineum.*

MATERIALS AND METHODS

Sourcing plant extracts

This study was done in Kenya and Eswatini from 2020 to 2021. The selection of the plants to be used was influenced by their known medicinal properties as well as availability (Abdel-Rahim et al., 2016; Berhanu and Babele, 2020; Carpinel and Alonso, 1999; Hasan et al., 2019; Jeruto et al., 2016; Okello and Kang, 2019). The selected plants were *Warburgia ugandensis, Carissa spinarum, Acacia nilotica, A. indica*, *M. azedarach* and *Senna didymobotrya*. The *M. azedarach* extracts were sourced from Eswatini and the other five plant leaves were sourced from the Jomo Kenyatta University of Agriculture and Technology’s (JKUAT) botanical garden, in Kenya.

Plant extract preparation

The plant crude extract preparation was done by cleaning the leaves with sterile water, drying them and cutting them into small pieces using a blender. Then 50 g of each leaf sample was heated at 80°C in 250 ml of sterile water in a 500 ml Erlenmeyer flask for 30 min. The crude leaf extracts were then filtered using Whatman No. 1 and stored at 4°C (Velu et al., 2017).

Biosynthesis of nanoparticles

To synthesize the AgNPs, 1 mM of silver nitrate was formulated by adding 0.167 g of silver nitrate into 1 L of distilled water. The mixture of the silver nitrate and the plant’s crude extract was made

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at an optimised ratio and kept in darkness, to prevent photo-reduction of the silver, at 28^{o}C in a 150rpm shaking incubator. The intensity of the colour which is indicative of nanoparticle formation was recorded between 200 and 800 nm on a UV-Vis spectrophotometer using the Flight Deck, Jenway Model 6800 Spectrophotometer (Velu et al., 2017).

Optimizing the nanoparticles

The b-AgNPs were optimised under different reaction conditions which included leaf extract reaction volume (2, 3, 4, 5, 6, 7, 8, and 9 ml) and the duration of incubation of the AgNPs in darkness which was varied at 0, 2, 4, 12, 24, 48 and 72 h (Houllou et al., 2019). While optimizing each parameter, the other parameters were kept constant.

The b-AgNPs were isolated from the optimized mixture by centrifugation at 12 000 rpm for 20 min. The pellet was then purified using distilled water and washed twice to ensure better separation of free entities from the AgNPs. The b-AgNPs were kept at -20°C for 24 h, moved to -80°C to be kept for 48 h, and then they were lyophilized and used for further characterization (Velu et al., 2017).

Characterizing the nanoparticles

UV-Vis Spectra analysis

A sample (1 ml) of the suspension was collected periodically to monitor the completion of bio-reduction of Ag+ in an aqueous solution. The UV-Vis spectrum of the solution was measured between wavelengths 200 and 800 nm using the Jenway Model 6800 Spectrophotometer Flight Deck with a resolution of 1 nm (Sanchooli et al., 2018).

FTIR analysis

The nanoparticle characterization included ascertaining the active biomolecules responsible for the reduction; capping and stabilising by Fourier transform infrared (FTIR) spectrometer model 8400, Shimadzu. For the FTIR analysis, the dried b-AgNPs were added to FTIR-grade potassium bromide (KBr) in 1: 100 ratios and observed in the range of 4000 to 400 cm^{-1} (Mondal et al., 2020).

TEM analysis

Analysis to determine the morphology, size and shape of the nanoparticles was done using the JEM-2100 Electron Microscopy. The TEM sample grid with a continuous silicon oxide film was prepared. The sample grid was then derivatized by exposing the silicon oxide to 10 µl of aminopropyldimethylethoxysilane solution. The b-AgNPs were then citrate-stabilized for them to have a negative charge to be attracted to the positively charged TEM surface grid (Bonevich and Haller, 2010).

Collection and identification of the fungus

The smut-infected plants were identified at the Eswatini Sugar Association's experimental plots at Nsoko. Visible sori were cut from the infected sugarcane plants and bagged to prevent any spread to healthy plants. These spores were rinsed three times with distilled water and cultured in potato dextrose agar (PDA). The plates were incubated for fourteen days in darkness at 28°C (Cui et al., 2020; Singh et al., 2005). To purify the cultures, single colonies were transferred onto new plates and incubated in darkness at 28°C (Que et al., 2014).

Fungal genomic DNA was extracted using a Zymo Fungal/Bacterial Genomic DNA Extraction Kit (Inqaba Biotech, South Africa) following the manufacturer's instructions. The quality and concentration of DNA were analysed by 1% agarose gel electrophoresis and by using a nanodrop spectrophotometer.

To verify the identity of the fungus, the extracted DNA was amplified in conventional PCR using the *bE*4 (5'-CGCTCTGGTTCATCAACG - 3') and *bE*8 (5'-TGCTGTCGATGGAAGGTGT - 3') primers that are specific for *S. scitamineum* (Zhang et al., 2015).

Conventional PCR amplification was carried out in a 25 μL volume containing 1 μL DNA, 12.5 μL of 2x OneTaq master mix, 0.5 μL of each of the upstream and downstream primers and 10.5 μL of water. The PCR amplification was performed following a thermal cycling programme of 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30 s, and 68°C for 40 s; and a final extension at 72°C for 5 min.

The PCR amplicons were checked for quality on a 1% agarose gel electrophoresis and then documented. A negative control sample only contained the master mix with no DNA template.

Screening of b-AgNP for antifungal activity

The silver nanoparticles that were produced from the crude extracts from the different plants were evaluated to select the b-AgNPs that had the highest antifungal activity against *S. scitamineum*. Fungal spores suspended in sterile water were spread onto PDA media using a sterile swab and then incubated for 24 h at 28°C. The disc method was used, with the standard antifungal nystatin (100 µg) as a positive control and distilled water as a negative control (Alyousef et al., 2019; Al-zubaidi et al., 2019; Hameed et al., 2015; Khan and Javed, 2021; Medda et al., 2015). The synthesised b-AgNPs were dissolved in distilled water and each b-AgNP treatment was evaluated at 2.5, 5 and 10 mg/ml in three replicates.

Data analysis

The antifungal efficacy of the various b-AgNPs was evaluated by measuring the zones of inhibition. Data were subjected to ANOVA and means were separated at P = 0.05.

RESULTS

Biosynthesis of silver nanoparticles

The reduction of the silver nitrate, upon mixing it with the crude extract, was seen by the colour change from light pale into dark brown (Figure 1).

Characterization of the b-AgNPs

UV-Vis spectroscopy

The analysis showed absorption peaks for the b-AgNPs that were made from the different botanicals at a range between 340 and 450 nm. To optimise the amount of crude leaf extract that was added to the 1 mM silver

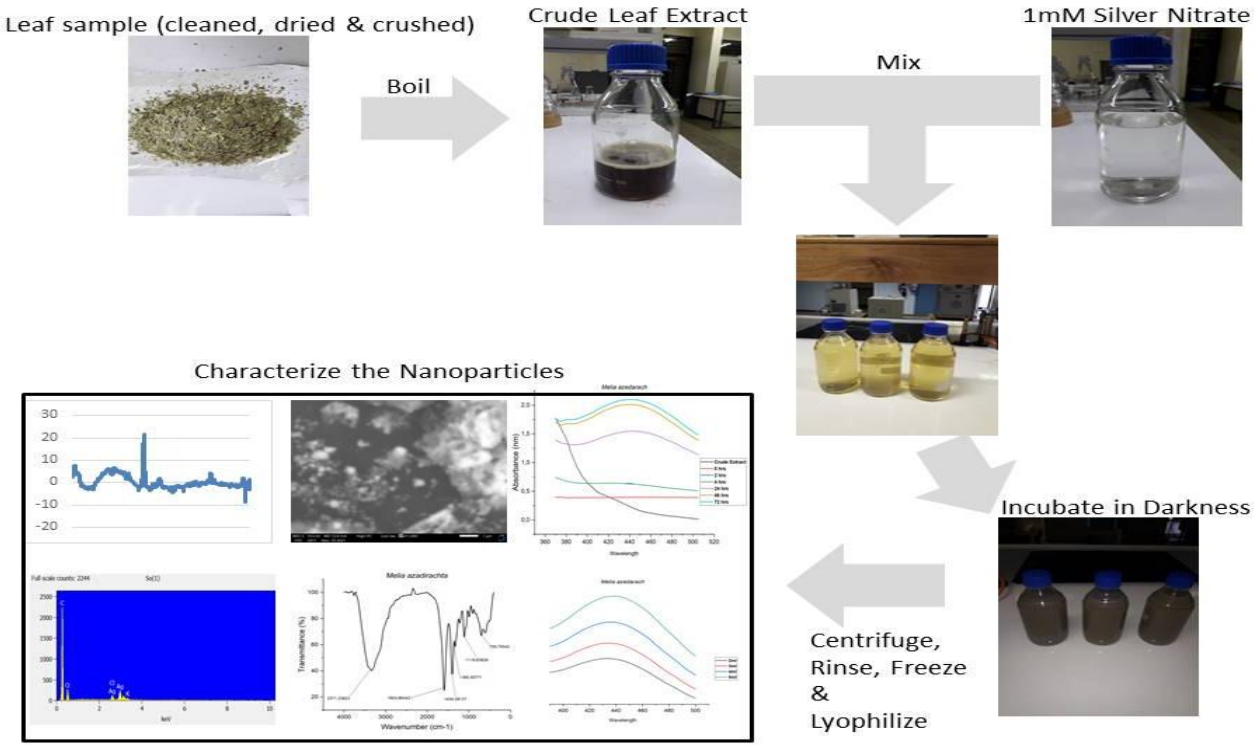

Figure 1. The biosynthesis of silver nanoparticles: by mixing the crude leaf extract with silver nitrate, incubating the mixture, centrifuge and lyophilise the AgNPs and then characterise the produced b-AgNPs
Source: Authors

nitrate ($AgNO^3$) to reduce it to AgNPs; 5 ml was added for *A. indica* and *Melia azedarach*, while 4 ml was added for *A. nilotica* and *S. didymobotrya,* 3 ml was added for *C. spinarum* and *W. ugandensis* and the plasmon peaks were observed at 430, 435, 385, 385, 335 and 385 nm, respectively (Figure 2). When optimizing for the required incubation period of the mixture; *A. indica, A. nilotica, M. azedarach* and *W. ugandensis* required to be incubated for 72 h, while *S. didymobotrya* required to be incubated for 48 h and *C. spinarum* required 24 h, and the plasmon peaks were observed at 390, 385, 440, 390, 395, and 390, respectively (Figure 3).

Fourier transform infrared (FTIR) analysis

The FTIR analysis of the b-AgNPs (Figure 4) shows the bands that correspond with the biomolecules responsible for the reduction of $AgNO_3$ to nanoparticles. The bands were observed at 3355 to 3402 cm^{-1}, 1595 to 1605 cm^{-1}, 1404 cm^{-1}, 1200 to 1300 cm^{-1}, 995 to 1118 cm^{-1}, and at 500 to 720 cm^{-1}.

TEM analysis

The sizes of the b-AgNPs that were synthesized using the various botanicals varied between 3 and 70 nm. *C. spinarum* produced AgNPs with the size range of 3 to 33 nm, *M. azedarach* produced 9 to 70 nm, while *A. indica, A. nilotica, S. didymobotrya* and *W. ugandensis* produced 14 to 53 nm, 14 to 52 nm, 6 to 35 nm and 12 to 53 nm, respectively (Figure 5 and Table 1). The shape and surface texture of the b-AgNPs was consistently spherical and smooth for all the nanoparticles (Table 1).

Verification of the fungi

To verify the identity of the fungus, the collected samples were screened by conventional PCR using the *S. scitamineum* specific primers *bE*4 and *bE*8. The samples produced an amplicon of 459 bp which corroborated the results by Izadi and Moosawi-jorf (2007).

Screening of nanoparticles for antifungal activity

The evaluation of the antifungal activity of the different b-AgNPs showed that *C. spinarum* and *M. azedarach* had higher inhibition zones, but *C. spinarum* was shown to be superior at both 5 and 10 mg/ml (Figure 6). *A. indica* recorded the least antifungal activity at all the concentrations, while the other b-AgNPs had a moderate

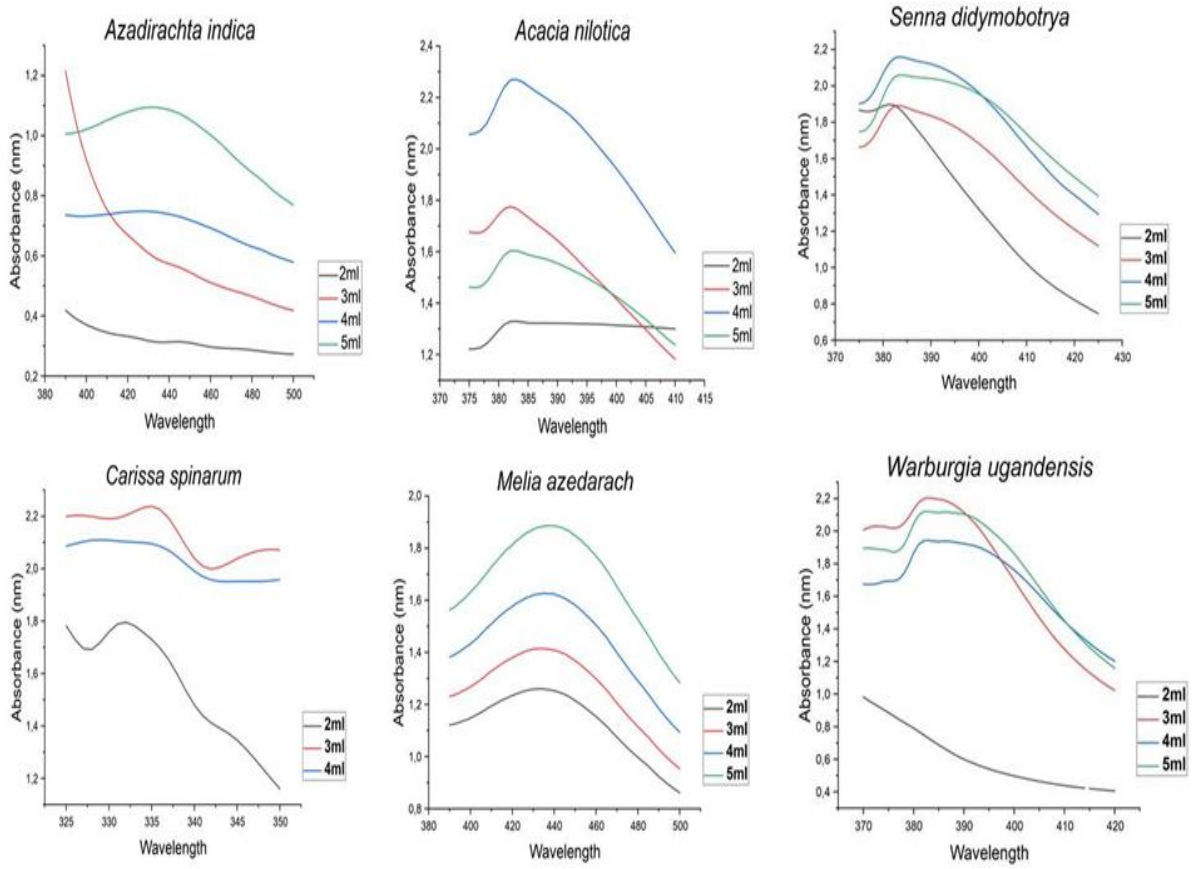

Figure 2. The optimization of the b-AgNPs by varying crude extract amounts of the six plants, observing the absorbance at a wavelength from 200 to 800 using a UV-Vis spectroscopy.
Source: Authors

effect.

DISCUSSION

The colour change upon mixing the silver nitrate with the crude extract is indicative of the formation of the nanoparticles by the reduction of the $AgNO_3$ by the crude leaf extract to form b-AgNPs (Alyousef et al., 2019; Sharma et al., 2014; Velu et al., 2017). The colour change is due to the occurrence of the Surface Plasmon Resonance (SPR) phenomenon which is caused by the interaction of the conduction electrons of the silver nanoparticles (Sharma et al., 2014; Vala et al., 2021). The phytochemicals (lipids, proteins, polyphenols, carboxylic acids, saponins, amino acids, polysaccharides and enzymes) present in plants are used as reducing, capping and stabilising agents (Chouhan, 2018).

During the synthesis of the b-AgNPs, the amount of crude extract that was added as well as the incubation period were optimised by UV-Vis spectroscopy. The optimization process produced absorption peaks for the b-AgNPs at a range between 340 and 450 nm and confirms the formation of the nanoparticles, which is consistent with the findings of Alyousef et al. (2019), Sanchooli et al. (2018) and Masum et al. (2019). Namratha and Monica (2013) reported the range of the peaks that indicate the formation of nanoparticles to be observed between 350 and 550 nm. The various botanicals required varying amounts of the leaf extract as well as incubation periods to achieve the optimum formation of the b-AgNPs. This variation could be caused by the changeable levels of phytochemicals that are contained in the different botanicals. This variation of phytochemicals could have resulted in a variable reduction rate of the silver nitrate as well as the variable capping and stabilizing of the nanoparticles (Chouhan, 2018).

The FTIR analysis of b-AGNPs validates the activity of biomolecules that are in charge of the reduction and stabilization of the b-AgNPs (Khatoon et al., 2017; Mondal et al., 2020). The analysis shows the bands between 3355 and 3402 cm^{-1} which correspond to N-H stretching of the proteins' secondary amide. The peaks at 1596-1605 cm^{-1} indicate stretch vibrations for the –C=C- bond, whilst the Benzene ring C=C and C-C are shown

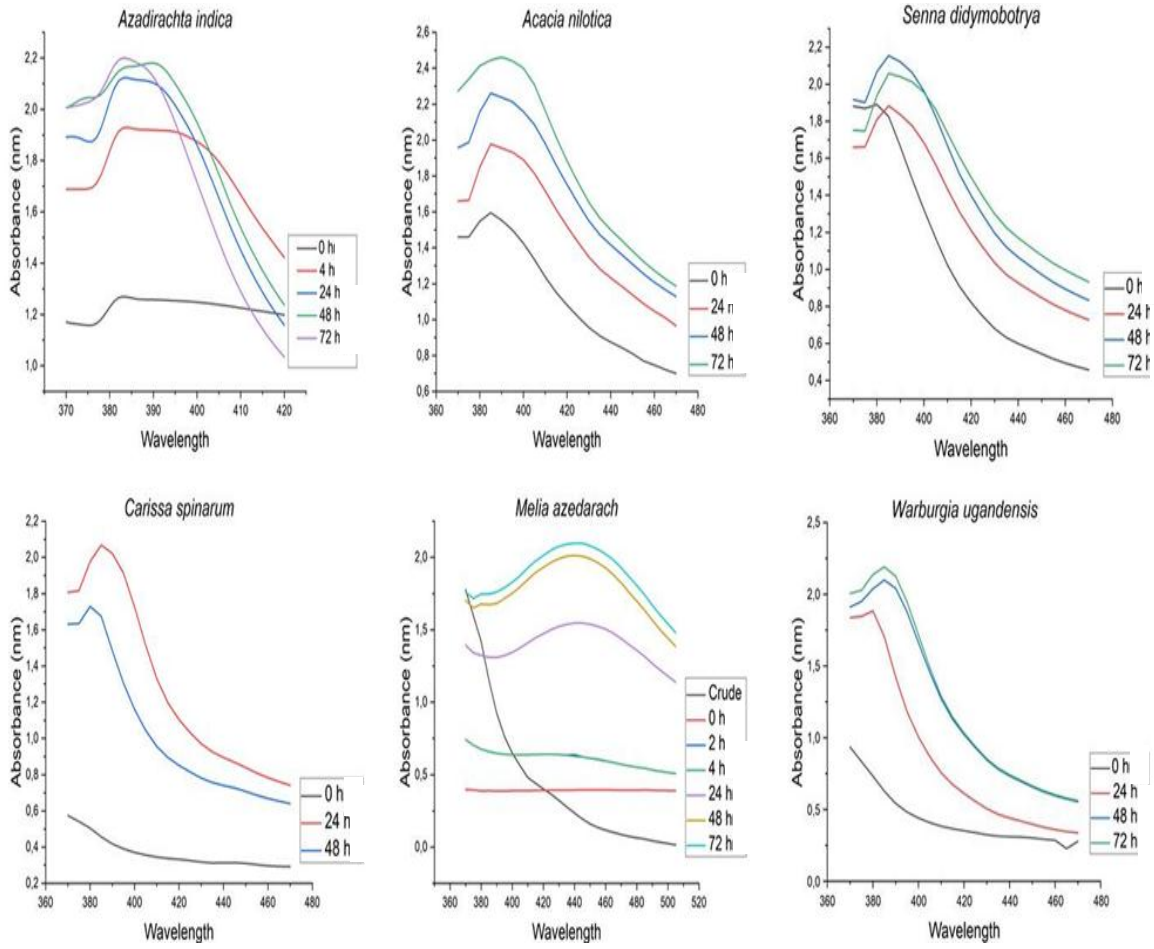

Figure 3. The optimization of the b-AgNPs by varying the incubation period, observing the absorbance at a wavelength from 200 to 800 using a UV-Vis spectroscopy.
Source: Authors

by peaks at 1404 cm^{-1}. The C-H bond in the pyridine ring appears at 1200-1300 cm^{-1} and the C-OH phenols appear at 995-1118 cm^{-1}. The peaks at 500-720 cm^{-1} show the presence of the AgNPs (Al-zubaidi et al., 2019). The synthesised nanoparticles were surrounded by proteins and other functional groups such as terpenoids. These results indicate the strength of the carbonyl groups from the proteins and amino acids to bind with metal, thereby capping the AgNPs. The presence of the reducing sugars could indicate their responsibility in reducing the $AgNO_3$ to AgNPs and stabilizing the AgNPs (Khatoon et al., 2017).

The TEM analysis was able to determine the sizes, shapes and texture of the b-AgNPs. Nanoparticles, by their definition, should range between 1 and 100 nm (Vala et al., 2021; Mondal et al., 2020). Their nano-scale size, morphological substructure and shape are of great importance as they give the AgNPs the physicochemical properties that suit them for their multiple applications (Chouhan, 2018; Khatoon et al., 2017). The size and shape of the *A. indica* AgNPs were shown to be consistent with the sizes that are documented by Firdhouse and Lalitha (2015), Khatoon et al. (2017) and Namratha and Monica (2013) (Table 1). The synthesis and characterisation of AgNPs made from other botanicals had not been documented before this study.

The fungal isolate was positively verified by conventional PCR using the *S. scitamineum* specific primers *bE*4 and *bE*8 (Zhang et al., 2015). All the b-AgNPs had an inhibitory effect on the growth of the fungus *S. scitamineum*, but with varying efficacies which could be due to the difference in phytochemicals that reduce the silver nitrate to nanoparticles among the botanicals (Hussain et al., 2019). This was observed by the formation of inhibition zones in all the b-AgNP treatments. The positive inhibition of the growth of the fungus *in-vitro* warrants further *in-vivo* studies.

Conclusion

In this study, biosynthesized silver nanoparticles (b-

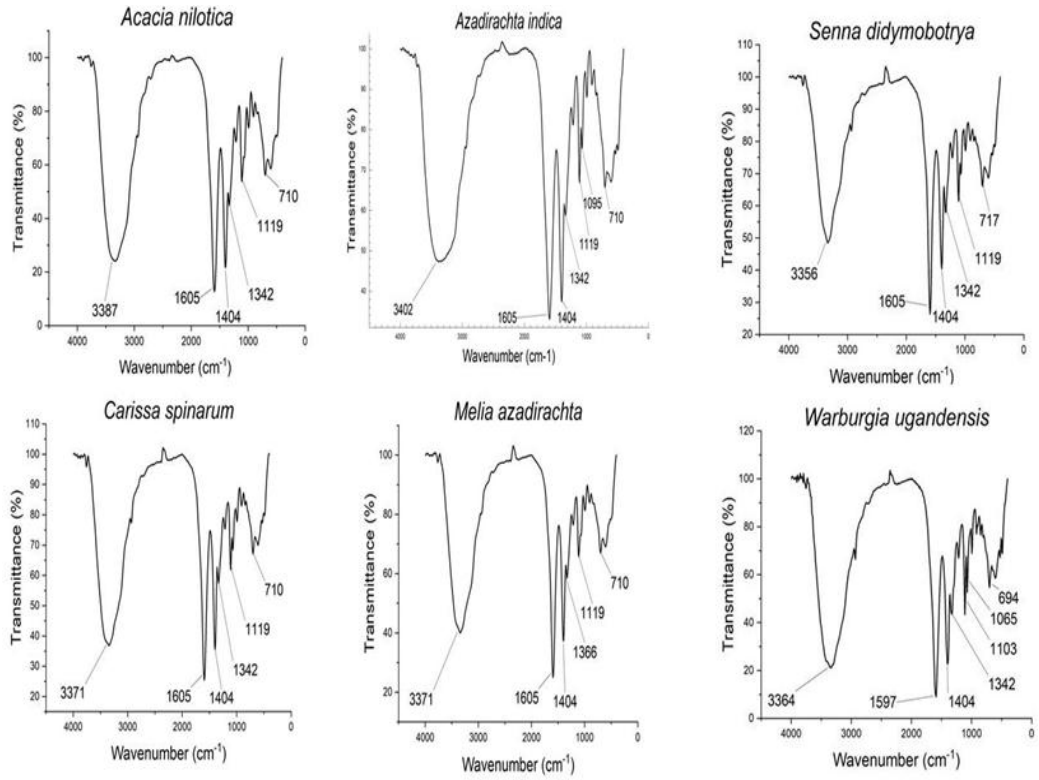

Figure 4. The FTIR results indicating the presence and sites (bands) of the biomolecules that are responsible for reducing the $AgNO^3$ to b-AgNPs as well as those responsible for capping and stabilising the AgNPs
Source: Authors

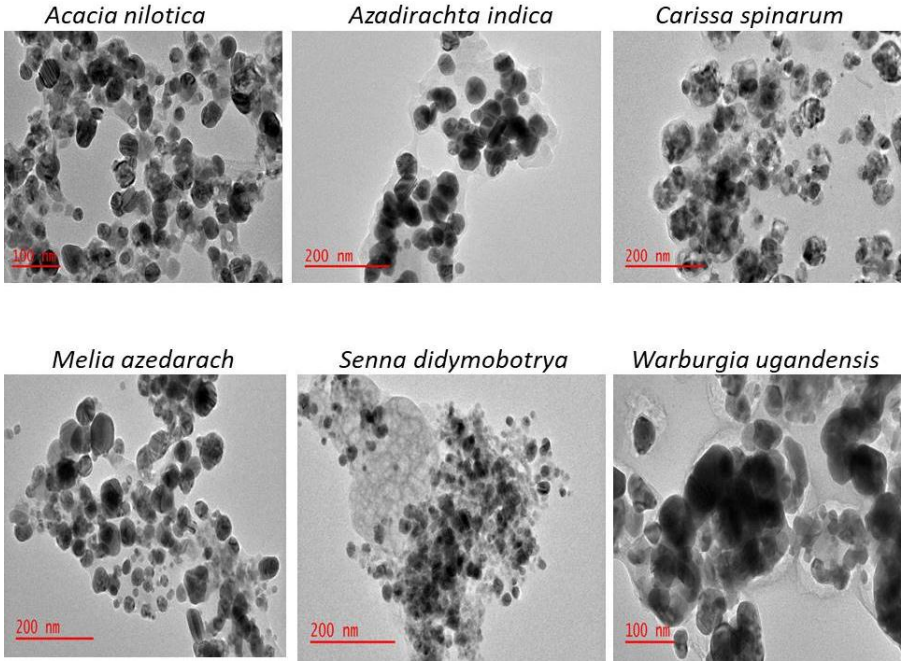

Figure 5. The TEM results indicating the sizes and shapes of the b-AgNPs that were produced using crude extracts from the different medicinal plants.
Source: Authors

Table 1. The size ranges, shapes and surface textures of the b-AgNPs that were produced using the six medicinal plants as observed by the TEM.

b-AgNPs	Size range (nm)	Shape	Surface texture
Acacia nilotica	14 - 52	Spherical	Smooth
Azadirachta indica	14 - 53	Spherical	Smooth
Carissa spinarum	3 - 33	Spherical	Smooth
Melia azedarach	9 - 70	Spherical	Smooth
Senna didymobotrya	6 - 35	Spherical	Smooth
Warburgia ugandensis	12 - 53	Spherical	Smooth

Source: Authors

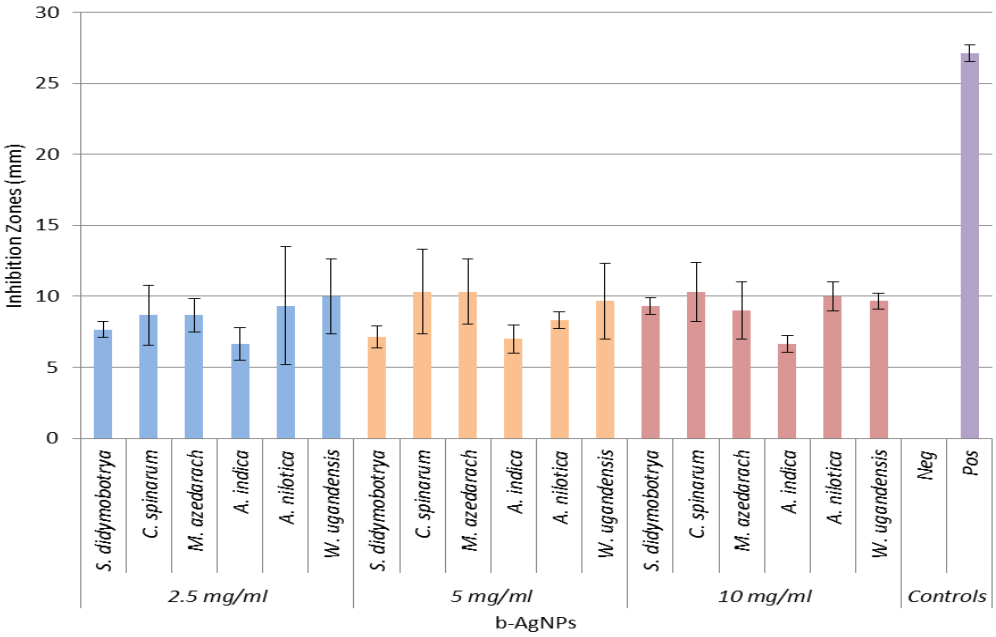

Figure 6. The antifungal effect of the b-AgNPs measured by their inhibition zones when tested in-vitro on *Sporisorium scitamineum* at 2.5, 5 and 10 mg/ml with nystatin as a positive control and distilled water as a negative control
Source: Authors

AgNPs) of varying sizes were successfully synthesized using *A. nilotica, C. spinarum, S. didymobotrya, W. ugandensis, M. azedarach* and *A. indica.* The synthesized nanoparticles were all spherical in shape, smooth in texture, and had a size range between 3 and 70 nm as indicated by the TEM, FTIR and UV-Vis characterization. All the biosynthesized nanoparticles showed antifungal effects against the fungus *S. scitamineum in-vitro. C. spinarum* and *M. azedarach* exhibited the highest antifungal activity, while *A. indica* showed the least.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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