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Antibacterial activities and biosynthesis of nanoparticles using hemp extracts

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Background: The use of plants in the biosynthesis of nanoparticles is a fast-growing technique and has gained much interest from researchers over the years.

Aim: This study reported the utilisation of leaf extracts of *Cannabis sativa* L. (hemp) for the biosynthesis of silver nanoparticles (AgNPs).

Methods: In this study, techniques such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), energy-dispersive X-ray (EDX) microanalysis and Fourier transform infrared (FT-IR) were carried out for the synthesis and characterisation of nanoparticles. The antibacterial efficacy of the synthesised nanoparticles was evaluated by using the agar diffusion and macrobroth dilution methods. The antibacterial properties of the biosynthesised AgNPs were evaluated against both Gram-negative and Gram-positive bacteria.

Results: The formation of AgNPs was confirmed by colour change in plant extracts and further characterised by Ultraviolet-visible (UV-Vis) spectrophotometry TEM, SEM, EDX and FT-IR analyses. Fourier transform infrared analysis revealed the efficient capping and stabilisation properties of these particles and the nature of the capping agent. Silver nanoparticles prepared from leaf extracts showed effective antibacterial activity against *Escherichia coli*.

Conclusion: Biosynthesised AgNPs showed a broad-spectrum antimicrobial susceptibility range and therefore represent promising antimicrobial agents. This is the first reported study for hemp leaf extracts and contributes to the environmentally friendly and cost-effective technique of biosynthesising nanoparticles for drug development.

Contribution: This study contributes to the current medicinal properties of cannabis. Furthermore, it reflects that cannabis can also be utilized at a nanoscale with effective antibacterial efficacy.

Keywords: biosynthesis; silver nanoparticles; antimibacterial activity; hemp; Cannabinaceae.

Introduction

Nanotechnology, especially nanoparticle synthesis, has emerged as a new area of biotechnology because of its multiple applications (Erjaee, Rajaian & Nazifi 2017). The 'nano' nature of the particles implies a substantially small size (1 nm – 100 nm) and an increased ratio of surface area to volume (Reda et al. 2019). Vadivu et al. (2017) defined nanotechnology as 'the synthesis, characterisation, exploration and application of nanosized materials for the development of science'. In addition, nanoparticles have distinguishable characteristics when compared with the original form of the same material (Vadivu et al. 2017). Nanosized materials are sought after because of their mechanistic, optical and biological attributes (Rajkuberan et al. 2017; Reda et al. 2019).

Metal nanoparticles (gold and silver) possess antibacterial activity (Mittal, Chisti & Banerjee 2014). Their investigation further elaborates other noble metals such as platinum and palladium that are already incorporated into personal hygiene products for their effectiveness in antibacterial activity. Gold is known to be compatible with biological material. The nanoparticles synthesised from biological material have had success in eradicating bacteria and cancerous tissue (Jadoun et al. 2020; Teimuri-Mofrad et al. 2017). Silver nanoparticles (AgNPs) are described as being the most required in the field of medical technology because of their antibacterial and anti-inflammatory activities (Reda et al. 2019). Tiwary and Jha (2017) reported that silver also possesses antiviral, antifungal, anti-parasitic and antidiabetic activities. More so, silver compounds are effective antiseptics and are used in surgery as protective clothing, such as wound dressing and bandages (Gunasekaran et al. 2017; Tiwary & Jha 2017). Currently, AgNPs can be synthesised using reducing agents such as hydrazine and other toxic chemicals (Khan & Khan 2017). However, synthetic methods are expensive, noxious and environmentally hazardous (Chinnasamy et al. 2017; Iravani et al. 2014; Khan & Khan 2017).

In addition, Gunasekaran et al. (2017) stated that reaction of AgNPs with mammalian cells showed a reduced level of noxiousness. It has been reported that AgNPs are lethal to about 650 different types of microorganisms coupled with a fast reaction time of 30 min (Tiwary & Jha 2017). Furthermore, Khan and Khan (2017) reported that fungi, bacteria and plants can be used as biological tools for the inexpensive and simple production of AgNPs, referring to this process as 'nano-factories'. However, plant material is the most suitable substance for the green synthesis of nanoparticles (Jadoun et al. 2020; Reda et al. 2019). Plants naturally have compounds which may possess medicinal properties and these have been attributed to the phytochemicals, which are able to reduce, cap and stabilise Ag^+ ions (Jadoun et al. 2020; Khatoon, Mazumder & Sardar 2017; Maddila & Hemalatha 2017). Plants and their extracts are simple to maintain, eco-friendly, rapid, affordable, easily available and their stability can be controlled (Hafez et al. 2017; Vanlalveni et al. 2021; Verma et al. 2017).

Cannabis is the preferred designation of the plant species *Cannabis sativa* (*C. sativa*) L., *Cannabis indica* Lam., and of minor significance, *Cannabis ruderalis* Janisch (Madras 2015), belonging to the family Cannabaceae. *Cannabis sativa* is known by various common names worldwide: it is called 'dagga' in South Africa, whilst marijuana and hemp are well associated common names (The Plant List 2020). The species *C. sativa* is a well-known plant grown worldwide but was originally a native of western and central Asia (Ayenigbara 2012; Kuddas & Ginawi 2013). The genus *Cannabis* has been utilised commercially, medically, religiously, socially and agriculturally for over a century (Ayenigbara 2012). *Cannabis* yields more than 538 chemicals of various classes (Kuddas & Ginawi 2013). The most common classes are cannabinoids, hydrocarbons, terpenoids, fatty acids, amino acids, proteins, pigments, alkaloids, enzymes, glycoproteins and simple ketones (Ali et al. 2011). These plants are mostly exploited for their medicinal and pharmacological uses (Madras 2015). *Cannabis* is administered to patients suffering from cholera, epilepsy, tetanus and rabies (Ali et al. 2011). Whilst *Cannabis* is used for the treatment of specific human ailments such as sexually transmitted diseases, burns, leukoderma allergies, cuts and wounds, inflammation, scabies and smallpox, it also exhibits pharmacological uses because of its antidiabetic, anti-inflammatory, antimicrobial, antioxidant, anticancer and anti-obesity properties (Ali et al. 2011; Ayenigbara 2012). Over a long period, clinical research on *Cannabis* was restricted because of its illegality. However, *Cannabis* has been used worldwide for millennia, making it one of the oldest known medicinal plants. The study on the psychoactive effect of tetrahydrocannabinol (THC) has presented possibilities to exploit *Cannabis*-based products for medicinal use. The aim of this study was to biosynthesise AgNPs using *C. sativa* leaf extracts with the view of exploiting the drug development potential of the plant as an effective antibacterial agent.

Materials and methods

Plant material and preparation of extracts

The specimens were collected by Mr Thami Kunene, conservation officer at Ethekwini Municipality, and stored in the Medicinal Plant Laboratory at Mangosuthu University of Technology, Durban, South Africa. Methanol extracts of leaves were prepared using fresh samples (20 g), which were crushed in methanol (100 mL) with a mortar and pestle before being filtered through Whatman No.1 filter paper and stored at 40 °C for 14 days.

Preparation of 1 mMol silver nitrate solution

One molar silver nitrate ($AgNO_3$) stock solution was prepared by dissolving $AgNO_3$ (0.17 g) (BDH Chemicals Ltd., England) in distilled water (100 mL). A 1-mM solution was prepared by diluting 1 M solution (10 mL) in distilled water (90 mL). This solution was stored for further use in a dark bottle at room temperature (24 °C). The concentration of the extracts and $AgNO_3$ were 17 000 μg/mL and 9000 μg/mL, respectively.

Synthesis of silver nanoparticles

Aqueous and methanol extracts of leaves (5 mL each) were separately added to 1 mM $AgNO_3$ solution (45 mL) for reduction of Ag^+ ions. Synthesis of AgNPs occurred at room temperature (24 °C). The change in colour of the solution (which indicated the formation of the AgNPs) was observed and the time was recorded.

Characterisation of Cannabis sativa silver nanoparticles

Ultraviolet–Visible spectrophotometric analysis

The reduction of pure Ag^+ ions was monitored by using an ultraviolet–visible (UV–Vis) spectrophotometer (Spectrostar Nano BMG, Germany), where distilled water was used as blank. The reaction medium was analysed for its maximum absorption at a wavelength range of 220 nm – 600 nm and the corresponding peaks were recorded. The absorbance of the reaction medium was measured within 24 h.

Evaluation of pH levels

The pH levels of the different extracts were measured before and after bio-reduction using a pH meter (WS Instruments, pH 50+, Italy) and calibrated at a two-point calibration between four and seven. The pH readings of methanol and silver were also recorded.

Scanning electron microscopy analysis and energy-dispersive X-ray microanalysis

Scanning electron microscopy (SEM) analysis was performed using a Zeiss Ultra Plus field emission scanning electron microscope (FE-SEM, Germany) at 5 kV. Thin films of the sample were prepared on a carbon-coated brass stub by adding a drop of the sample onto the coverslip mounted on the stub. The sample was allowed to air-dry for 10 min. Samples on the stubs were then sputter coated with gold for approximately 20 min. The shape and morphology of the nanoparticle clusters were identified. Energy Dispersive X-ray Analysis (EDX) was used to identify the composition of the synthesised AgNP and to confirm the presence of silver. Energy-dispersive X-ray analysis was performed using a

Zeiss Ultra Plus FE-SEM with Aztec software (Oxford instruments, UK) at 5 kV.

Transmission electron microscopy analysis

Transmission electron microscopy (TEM) analysis was performed to characterise the size and shape of the synthesised AgNPs. A drop of the nanoparticle solution was placed on a formvar-coated copper grid and air-dried for 10 min. Images were viewed using the Joel TEM 1010 (Japan) at 200 kV.

Fourier transform infrared analysis

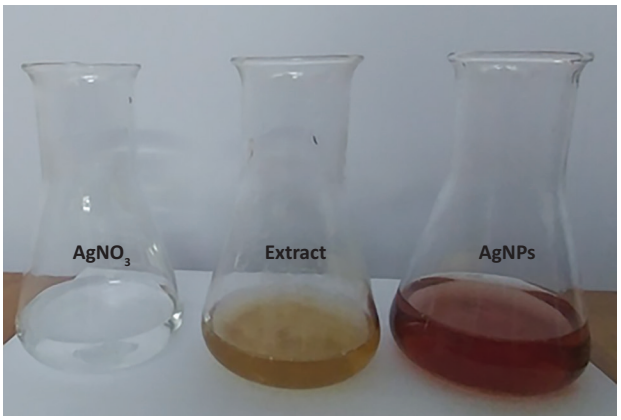
Fourier transform infrared (FT-IR) measurements were performed to identify the possible biomolecules responsible for reduction, capping and efficient stabilisation of AgNPs and the local molecular environment of the capping agents on the nanoparticles (Verma et al. 2017). Fourier transform infrared analysis of dried biomass after bio-reduction was carried out by removing any free residue from the capping ligand. The residual solution of 70 mL after reaction was centrifuged at 5000 rpm for 15 min and the supernatant was decanted and the pellet formed was dried in an oven at 50 °C. The dried nanoparticle was analysed to evaluate the bio-reducing and capping functional groups of the AgNPs. Infrared spectra of the crude extracts and their corresponding biosynthesised AgNPs were obtained using an Alpha-P ATR, FT-IR spectrophotometer (Bruker Optic, Germany).

Antibacterial assay

Preliminary antibacterial screening of the biosynthesised AgNPs were carried out against two Gram-positive reference bacteria, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* Rosenbach ATCC BAA-1683 (methicillin-resistant *S. aureus* [MRSA]) and four Gram-negative reference bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 31488, *Escherichia coli* ATCC 25922 and *Salmonella typhimurium* ATCC 14028). These bacteria were grown overnight in nutrient broth (Biolab, South Africa) at 37 °C in a shaking incubator (100 rpm). The bacterial concentration was adjusted to 0.5 McFarland's Standard with sterile distilled water using a DEN-1B McFarland densitometer (Latvia). Mueller-Hinton Agar (MHA) plates (Biolab, South Africa) were lawn inoculated with the prepared bacterial suspensions using a sterile throat swab and 5 μL of the extracts was spotted onto MHA plates. The plates were incubated at 37 °C for 18 h. Plates were read to determine antibacterial activity, denoted by clear zones in the area where the extracts were spotted. Based on the preliminary screening results, the minimum inhibition concentration (MIC) of the AgNPs was determined as the lowest concentration that resulted in no visible bacterial growth. The aqueous dispersions of AgNPs were serially diluted in twofold with water ranging from 0.1 μg/mL to 500 μg/mL) and 5 μL of each concentration was spotted on the lawn inoculated MHA plates and incubated at 37 °C for 24 h. The crude extracts and $AgNO_3$ solution served as controls.

Results and discussion

This was the first experimental study on the biosynthesis of AgNPs using leaf extracts of *C. sativa*. The findings of this study were confirmed and compared with previous studies (Reda et al. 2019; Singh et al. 2018; Vanlalveni et al. 2021). A colour change in the reaction mixture is the first step that indicates that nanoparticles have been biosynthesised (Moodley et al. 2018; Reda et al. 2019). In this study, the colour changed gradually within the first 30 min from yellowish brown to dark brown after addition of the $AgNO_3$ to the leaf extract (Figure 1). Colour change after bio-reduction was an indication of the surface plasmon resonance (SPR) excitation in AgNPs and a brown-coloured solution was often visible (Singh et al. 2018). The SPR of AgNPs showed a peak centred near 430 nm and 450 nm, which corresponded to the absorbance of AgNPs reported in other studies using medicinal plants (Figure 2) (Govindappa et al. 2016). Ultraviolet–visible analysis was in accordance with earlier studies using cannabinoids derived from *C. sativa* (Hazekamp et al. 2005). The efficiency of the protocol used in the present study was evidenced by the fact that complete reduction occurred at room temperature (24 °C).



$AgNO_3$, silver nitrate; AgNPs, silver nanoparticles.

FIGURE 1: Colour change observed in the biosynthesis of silver nanoparticles with methanol leaf extract.

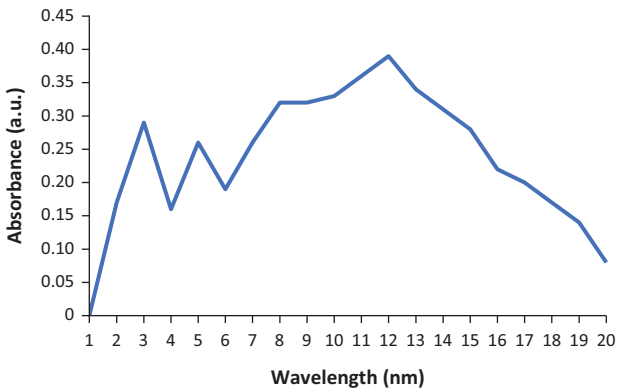


FIGURE 2: Ultaviolet–visible absorption spectra of silver nitrate with 1 mL *Cannabis sativa* leaf extracts 24 h after synthesis.

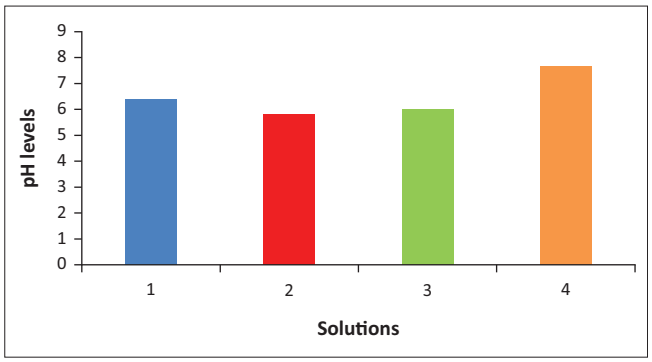


FIGURE 3: The pH levels of the different solutions before and after synthesis of silver nanoparticles (AgNPs): (a) methanol, (b) silver nitrate, (c) leaf crude methanol extract and (d) AgNPs.

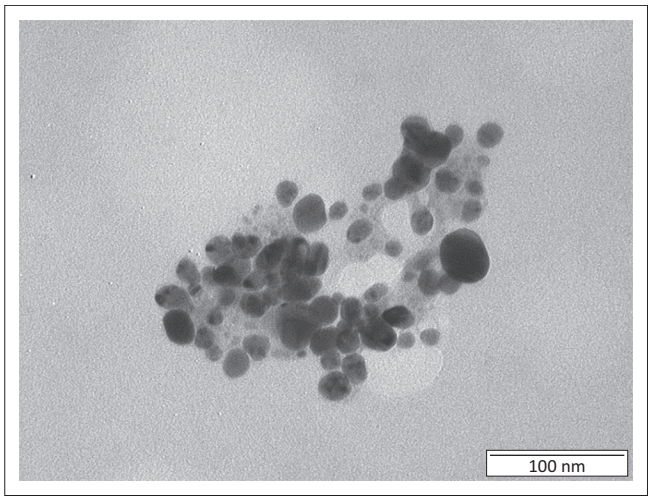


FIGURE 4: Transmission electron microscopy micrograph of silver nanoparticles synthesised using leaf extract of *Cannabis sativa* (Scale 200 nm at 200 kV).

The pH levels for methanol (pH 6.5), $AgNO_3$ solution (pH 5.8) and the crude extract (pH 6) appeared to be neutral (Figure 3a, b and c). The pH levels of AgNPs (pH 7.7) were slightly more alkaline after bio-reduction. The pH levels of AgNPs using *C. sativa* are reported for the first time in this study. This study agrees with previous studies that showed aqueous mediums to be more effective in biosynthesis because pH affects the production and stability of the nanoparticles (Kumari et al. 2016).

The TEM analysis clearly illustrated that the particles are spherical in shape and appear clustered (Figure 4), ranging in size from 20.31 nm to 24.7 nm. It was reported that the shape of metal nanoparticles can considerably change their optical and electronic properties (Singh et al. 2018). Analysis of aqueous silver nanoparticle samples provided unequivocal evidence that the prepared AgNPs were spherical in shape. Furthermore, the particles were observed to be stable and dispersed, even within aggregates. This finding is in agreement with previous reports for AgNPs derived from plant extracts (Khan & Khan 2017; Reda et al. 2019). As a result, the development of silver nanostructures was validated. Interestingly, a previous study on the influence of nanoparticle shape on bioactivity suggested that spherical shaped AgNPs display superior antimicrobial activities when compared with rod-shaped AgNPs (Reda et al. 2019).

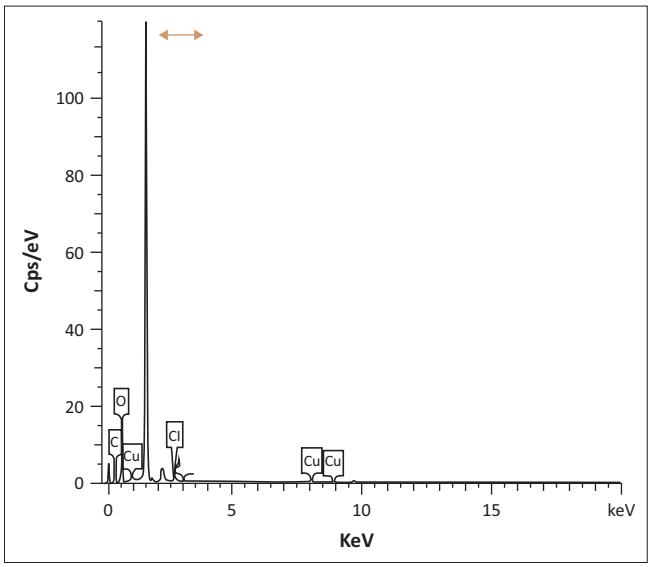


FIGURE 5: Energy-dispersive X-ray microanalysis spectra recorded after formation of silver nanoparticles using *Cannabis sativa* methanol leaf extract.

However, recent studies have suggested that the bioactivity of AgNPs occurs in a size-dependent manner, with smaller particles exerting better bioactivities than larger ones (Jadoun et al. 2020). The particles produced here were of average size based on previous plant synthesised particles and no conclusive inferences could be made on its bioactivity potential (Reda et al. 2019; Yadav et al. 2016). However, the outcomes were in the range of other reports (Singh et al. 2018; Velammal, Devi & Amaladhas 2016).

Energy-dispersive X-ray spectroscopy analysis of AgNPs demonstrated a strongly well-defined silver signal at 1.5 kV along with weak carbon, oxygen and nitrogen peaks – with the latter weaker signals probably representing surface biomolecule capping structures originating from the leaf extracts (Figure 5). Considering that this is a first-hand report on *C. sativa*, however, results were reported previously in other plant species (Hafez et al. 2017; Vadivu et al. 2017) and believed to originate from biomolecule capping structures (Reda et al. 2019).

Fourier transform infrared analysis was carried out to characterise the chemical functions in the organic extract responsible for the stabilisation of AgNPs (Figure 6). The sharp peaks between 3610 cm^{-1} and 3645 cm^{-1} confirmed the presence of free –OH bonds in the extract. The prominent peaks at 2916 cm^{-1} and 2849 cm^{-1} can be attributed to the terminal methyl groups present. Whilst small peaks near 1650 cm^{-1} are strongly likely to represent the C = O and C = C stretches, sharp peaks near 1550 cm^{-1} and 1507 cm^{-1} maybe be because of the presence of nitro groups (N–O stretch). Another peak at 1459 cm^{-1} is like those of a –C-H stretching, whilst peaks at 1188 cm^{-1} and 1026 cm^{-1} are typical characteristic of a C–O stretching attached to the presence of a tertiary alcohol group.

These functional groups in the extract are responsible for the reduction of precursor metal salt and the stabilisation of the

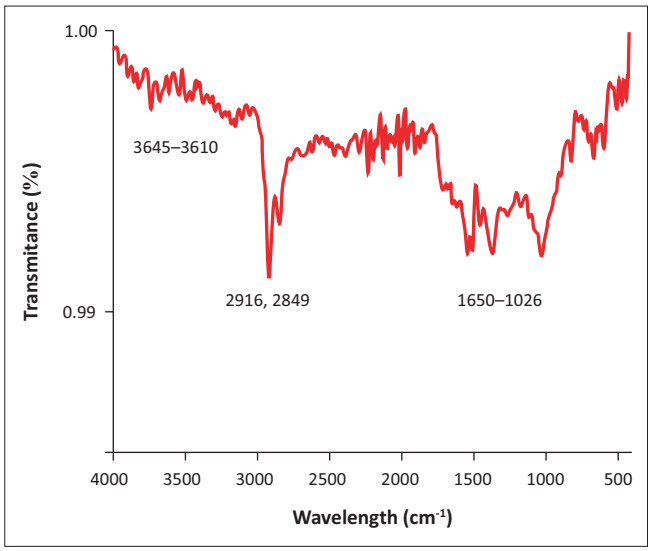


FIGURE 6: Fourier transform infrared spectra of *Cannabis sativa* methanol extract from the leaves after silver nanoparticles biosynthesis.

TABLE 1: Minimum inhibitory concentration of biosynthesised silver nanoparticles.

Microorganism	Minimum inhibitory concentration (μg/mL)		
	Extract	$AgNO_3$	AgNPs
Escherichia coli	15.62	250	1.95
Staphylococcus aureus	31.25	250	7.81
Klebsiella pneumoniae	31.25	250	3.91
Salmonella typhimurium	62.50	250	15.62
Methicillin-resistant *Staphylococcus aureus*	62.50	250	31.25
Pseudomonas aeruginosa	62.50	250	31.25

$AgNO_3$, silver nitrate; AgNPs, silver nanoparticles.

AgNPs formed. The hydroxyl and carboxyl groups act as a reducing agent and depended on the modified nature of the extract, the size and morphological characteristics of the nanoparticles (Devi & Selvan 2017; Maddila & Hemalatha 2017; Singh et al. 2018). Furthermore, the long-chain organic molecules would also help in the stabilisation of the AgNPs formed. These results are in agreement with previously reported studies carried out for reduction and stabilisation of AgNPs with plant extracts (Rajkuberan et al. 2017; Singh et al. 2018).

The AgNPs synthesised from *C. sativa* leaves in this study displayed antibacterial activity against both the Gram-negative and Gram-positive bacteria (Table 1). The $AgNO_3$ solution showed reasonable antibacterial activity (MIC: 250 μg/mL) against all the test bacteria. The synthesised nanoparticles and the crude extracts were active against all the bacteria tested at varying concentrations (1.95 μg/mL – 250 μg/mL). Silver nanoparticles displayed a higher antibacterial activity against the Gram-negative bacteria in comparison to the Gram-positive bacteria. Among the Gram-negative bacteria tested, AgNPs had the highest activity against E. coli, which was consistent with previous research (Ali et al. 2011). Testing using Gram-positive bacteria revealed that AgNPs solutions exhibited highest antibacterial activity against *S. aureus* (MIC 7.8 μg/mL) and lowest activity against MRSA (31.2 μg/mL). The crude methanol extract of *C. sativa* leaves also displayed antibacterial activity, with the extract being most active against *E. coli* (MIC 15.6 μg/mL). It was evident that aqueous extract was more active against Gram-negative than Gram-positive bacteria. Results from previous studies supported the antibacterial potential of AgNPs obtained in this study (Ali et al. 2011; Singh et al. 2018, Vanlalveni et al. 2021).

Amongst the Gram-negative bacteria tested, AgNPs showed maximum activity against *E. coli* and the result was in accordance with previous studies (Ali et al. 2011). Overall, the effective inhibition of both Gram-negative and Gram-positive bacteria by AgNPs derived from *C. sativa* leaf extract was significant as it demonstrated their broad-spectrum antibacterial activity. It also indicated that the mode of action was not affected by the difference in membrane stabilities of the bacteria because Gram-positive bacteria contain a thick peptidoglycan layer whilst Gram-negative bacteria possess a rigid outer membrane structure composed of lipids and lipoproteins (Devi & Selvan 2017; Vanlalveni et al. 2021). The low antibacterial activity of the $AgNO_3$ solution inferred that the bacteria were less prone to silver salts and that *C. sativa* has antibacterial properties (Ali et al. 2011; Phanjom & Ahmed 2015). This was confirmed by the high antibacterial activity of the crude extracts when compared with the $AgNO_3$ solution. This study also revealed that the biosynthesised AgNPs were found to have higher antibacterial activities when compared with the crude extract. This is in accordance with previous studies that have reported better antibacterial activity of AgNPs than the crude extracts (Chandrappa et al. 2016; Reda et al. 2019; Singh et al. 2018). As in previous studies, this study revealed that plant extracts and silver in their nano form are biologically active and can be used as antibacterial agents in potential drug development (Moodley et al. 2018; Vanlalveni et al. 2021).

Conclusion

Silver nanoparticles were successfully synthesised using fresh *C. sativa* leaf extracts. A change in colour upon addition of the $AgNO_3$ confirmed the synthesis of AgNPs. Characterisation by UV-Vis, TEM, SEM and EDX microanalysis analyses further confirmed the reduction of Ag^+ ions. Fourier transform infrared spectrum revealed that the –OH and >-C = O groups present in the biomolecules were responsible for the stabilisation and reduction of the AgNPs. Silver nanoparticles and crude extracts had desirable antibacterial activity against both Gram-negative and Gram-positive bacteria. Biosynthesis of AgNPs using *C. sativa* extracts provides an environmentally friendly and cost-effective option in comparison to chemical and physical synthesising techniques. The results obtained in this study were reported first-hand for this species, thus enhancing the current knowledge of biosynthesised metal nanoparticles and the medicinal value of *C. sativa*. Significantly, biosynthesised AgNPs showed a broad-spectrum antimicrobial activity range and therefore represent promising antimicrobial agents with potential biomedical applications.

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Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

All authors contributed equally to this work.

Ethical considerations

This article followed all ethical standards for research without direct contact with human or animal subjects.

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Data availability

The authors confirm that the data created or analySed in this study are included in this manuscript.

Disclaimer

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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