Antibacterial and Antifungal Properties of Silver Nanoparticles Synthesised using Phytochemicals from *Zanthoxylum piperitum* Leaves

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Abstract

Silver nanoparticles (AgNPs) have recently emerged as a potential treatment for diseases caused by multidrug-resistant microorganisms. The high surface area-to-volume ratio of AgNPs could deliver high performance as an antimicrobial agent. The current study investigated the synthesis and antimicrobial properties of AgNPs fabricated using phytochemicals extracted from Zanthoxylum piperitum leaves (Japanese pepper). ZPLAg-NPs (4.25 mg/mL) were prepared using green chemistry procedures, and the successful synthesis was indicated by a maximum absorption band at 420 nm in a UV-Vis spectrum. The fabricated ZPLAg-NPs showed remarkable effectiveness against gram-negative and gram-positive bacteria. The antimicrobial efficacy was shown by minimum inhibitory concentrations (MICs) of 0.07 and 0.13 mg/mL for Staphylococcus aureus and Escherichia coli, respectively. The nanoparticles also yielded minimum bactericidal concentrations (MBCs) of 0.53 and 0.13 mg/mL for Staphylococcus aureus and Escherichia coli, respectively. Similarly, the ZPLAg-NPs showed efficacy against Candida albicans and Aspergillus niger, producing MICs of 1.06 and 0.13 mg/mL, respectively. MBCs of 1.06 and 0.53 mg/mL were displayed for Candida albicans and Aspergillus niger, respectively. The findings from this study demonstrated that ZPLAgNPs could serve as a potential microbial agent for treating some bacterial and fungal diseases. .

Keywords: *Zanthoxylum piperitum*, nanoparticles, antimicrobial, green chemistry

Introduction

To address rising bacteria resistance to various convectional antimicrobials, nanotechnology applications in medical domains have grown recently. Metallic nanoparticles show enhanced bioactivity against microorganisms due to their high surface area-to-volume ratio (Nandhini *et al.,* 2023). The property enables metallic nanoparticles to penetrate a cell wall of microorganisms and generate metals, which inactivate important enzymes to cause death. As a result, even at low concentrations, they exhibit exceptional antibacterial activity (Vidyasagar *et al.,* 2023). Silver nanoparticles have been the most widely used among metallic nanoparticles because of their low cost, cytotoxicity, and immunological reactions (Calderón-Jiménez *et al.,* 2017). Additionally, the nanoparticles promote non-drug resistance and exhibit more substantial antibacterial effects (Li *et al.,* 2021). As a result, silver nanoparticles are frequently utilised in the biomedical field for various applications, including medication delivery, medical imaging, and molecular diagnostics (Calderón-Jiménez *et al.,* 2017).

Different methods, such as biological, physical, and chemical methods, have been employed to synthesise silver nanoparticles (Calderón-Jiménez *et al.,* 2017). Among these, chemical synthesis is the most preferable method because of its simplicity, cheapness, and high efficiency. The technique also allows the control of nanoparticle sizes by optimising experimental conditions (Dhand *et al.,* 2016; Vidyasagar *et al.,* 2023). The nanoparticles with smaller sizes showed higher microbial performance compared to larger ones. However, some chemicals used for the chemical synthesis of nanoparticles are reported to be toxic and form hazardous by-products that affect the environment (Nie *et al.,* 2023). Consequently, harmful chemicals in synthesising silver nanoparticles are highly discouraged, and there is increased demand for green synthesis processes.

Green synthesis creates silver nanoparticles by employing phytochemicals from different plant extracts as potential reducing and capping agents (Hasan *et al.,* 2022). Terpenes, flavonoids, alkaloids, phenol ketones, aldehydes, amides, and carboxylic acids are just a few of the biomolecules found in plant extracts that can be used as efficient reducing and stabilising agents in the synthesis of silver nanoparticles (Dhand *et al.,* 2016; Vidyasagar *et al.,* 2023). The manufacture of silver nanoparticles using phytochemicals has the benefit of being inexpensive, readily available, and environmentally friendly. Various plant parts, including leaves, roots, stems, barks, and seeds, have been used to make silver nanoparticles (Hasan *et al.,* 2022; Vidyasagar *et al.,* 2023).

The *Zanthoxylum piperitum* plant, also known as the Japanese pepper, belongs to the Rutaceae family. It is a deciduous shrub or tree primarily found in the Korean Peninsula, China, and Japanese islands. The plant's pericarp is a spice with a distinctive aroma. The herb can also treat stomachaches, diarrhoea, moist cutaneous ulcers, and vomiting (Jong Moon *et al.,* 2001). The leaves of *Zanthoxylum piperitum* contain various compounds, including aromatic acids and flavonoids, which can be a good source of reducing and stabilising agents for the green fabrication of AgNPs. *Zanthoxylum piperitum* leaves are known to contain various compounds, such as aromatic acids and flavonoids, that can be a good source of reducing and stabilising agents for the fabrication of silver nanoparticles (Hieu *et al.,* 2014; Yamasaki *et al.,* 2022) . However, little information is available on using plant phytochemicals in manufacturing silver nanoparticles. The current study will report attempts to use compounds derived from *Zanthoxylum piperitum* leaves to synthesise silver nanoparticles with antimicrobial properties.

Experimental

Materials

Silver nitrate 99%, deionised water (18.2 MΩ.cm), *Zanthoxylum piperitum* leaves (ZPL) extract was used as reducing and capping agent.

Preparation of aqueous extract

Fresh *Zanthoxylum piperitum* leaves (Fig. 1) were gathered from the Mazimbu campus (SUA). Potential contaminants were removed by washing them with deionised water. The leaves were then oven-dried at 50°C to remove water. The dried leaves were ground to produce powder, which was boiled at 80°C in deionised water (0.05 g/mL) for 30 min. The mixture was filtrated, and a clear solution was used for the synthesis of ZPLAgNPs (Mwakalesi, 2023).

Figure 1: Photograph showing *Zanthoxylum piperitum* leaves

Phytochemical analysis

The aqueous extract solution prepared in 2.2 was qualitatively examined for phytochemicals, alkaloids, proteins, flavonoids, polyphenols, saponins, and phenols using standard methods as previously described (Dada *et al.,* 2018).

Synthesis of silver nanoparticles (ZPLAg-NPs)

The AgNO3 solution (0.001-0.04 M) was mixed with various amounts of the extract (5, 10, 20, and 30 mL), and the pH was then adjusted with NaOH (1 M) and HCl (1 M). The resulting mixture was shaken using a shaker at 100 oscillations per minute for 0 to 30 minutes at three different water bath temperatures (30, 40, and 50°C). The reaction was carried out in a dark condition to prevent the auto reduction of silver ions caused by light. The fabrication of ZPLAg-NPs was visually monitored by a colour change and using a 6715 UV-Vis spectrophotometer (JENWAY). UV-Vis spectra were reported as a function of reaction time, pH, extract amount, and silver nitrate concentration. The UV-Vis measurements were performed by continuous scanning from 300 to 700 nm using deionised water as a reference for baseline correction.

Antimicrobial study

Antimicrobial properties of silver nanoparticles were investigated by establishing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests. The microbes used in this study were selected gram-negative (Escherichia coli-ATCC 25922) and gram-positive (Staphylococcus aureus- ATCC 25923) bacteria, and (Candida albicans- ATCC 25923) and Aspergillus niger-ATCC 10231) fungi. Antimicrobial experiments were performed using silver nanoparticles synthesised using the optimised experimental conditions of 30 min reaction time, pH 5, 0.02 M AgNO3, 10 mL of plant extract, and 30 C reaction temperature.

Results and Discussion

Phytochemical analysis

The phytochemicals in plant extracts are used as reducing and stabilising agents in forming $AgNO_3$ (Dada *et al.,* 2019). Thus, chemical compounds contained in the plant extract used in the current study were examined in preliminary experiments. The results (Table 1) showed that the plant extract had phytochemicals belonging to tannins, saponins, flavonoids, and phenolic compounds. The presence of phenolic and flavonoids in *Zanthoxylum piperitum* leaves was previously reported (Jong Moon *et al.,* 2001). Similar compounds, such as saponins and flavonoids derived from plant extracts, were once used as effective reducing and stabilising agents for synthesising silver nanoparticles (Sahu *et al.,* 2016). Therefore, the *Zanthoxylum piperitum* leaf extract contains compounds with potential reducing and stabilising properties that can aid in the formation of silver nanoparticles. Thus, subsequent experiments used the aqueous plant extract to synthesise silver nanoparticles.

Table 1: Phytochemical analysis of *Zanthoxylum piperitum* leaves

Compound class	Inference
Phenolic	Present
Proteins	Absent
Tannins	Present
Flavonoids	Present
Saponins	Present
Glycosides	Absent
Steroids	Absent

Optical Observation and UV-Vis Spectrophotometry

The formation of silver nanoparticles involves a direct reaction between phytochemicals contained in the extract and silver ions. Thus, a colour change of the reaction mixture was used as a preliminary indicator for the formation of silver nanoparticles. The results (Fig. 2) showed that the reaction mixture changed colour from light yellow to brown due to the excitation of surface plasmon resonance (SPR) (Tanase *et al.,* 2020). UV-Vis spectrophotometry is a valuable technique that provides a deeper insight into optical properties that depend on silver nanoparticle size distribution and surface properties. The method was, therefore, used to confirm the formation of the silver nanoparticles. The findings (Fig. 3a) indicated that the silver nanoparticles displayed a strong surface plasmon resonance band (SPR) at 420 nm. The observed plasmon resonance band was attributed to collective oscillations of free electrons caused by strong interactions of silver nanoparticles with UV-light energy. Additionally, the observed absorbance spectrum was sharp and narrow in appearance, indicating the formation of spherical nanoparticles with a homogeneous distribution. According to Mie's theory (Hao *et al.,* 2004), a single symmetrical surface plasmon absorption band shows spherically shaped nanoparticles. The observed plasmon resonance band agrees with other previously reported silver nanoparticles (Mohapatra, 2014; Soltani *et al.,* 2018).

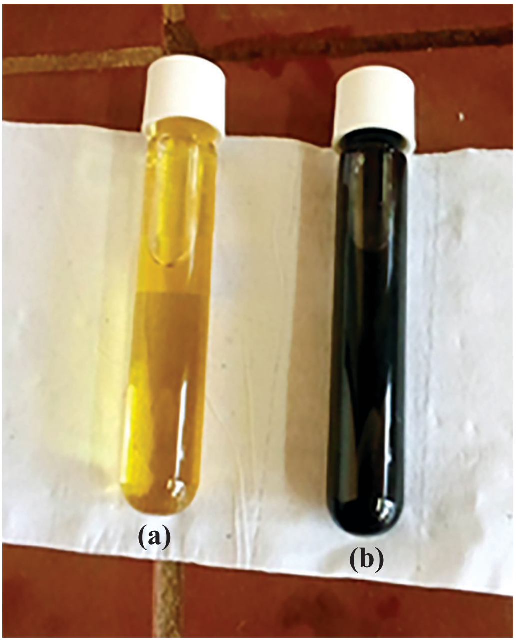

Figure 1: Optical images of aqueous plant extract (a) and colloidal silver nanoparticles (b)

Optimisation of experimental conditions

The influence of reaction time on the synthesis of silver nanoparticles was investigated at intervals of 10 from 10 – 40 minutes at room temperature (30°C). The findings presented in Figure 2(b) indicated that the nanoparticles started forming during mixing solutions (0 min). A slight increase in the formation of silver nanoparticles was observed as the reaction time increased from 0 to 20 min and decreased at 30 min. The increase in the formation of the nanoparticles indicates that more significant amounts of silver ions were converted to their metallic form as the reaction time increased. However, no further increase in the absorption band was observed when all the silver ions were converted entirely. Thus, the slight decrease in absorption at 30 min could result from the agglomerations of formed silver nanoparticles. The rapid formation of the nanoparticles observed in the current study indicates that the phytochemicals contained in the extract have high reducing power. The current study chose a reaction time of 20 min as the optimal reaction time needed for synthesising silver nanoparticles. Consequently, all subsequent experiments were performed using 20 20-minute reaction time.

Reaction mixture pH is among the factors affecting size and shape of silver nanoparticles (Handayani *et al.,* 2020). The pH affects the charges of the phytochemicals involved in reducing the stabilisation of silver nanoparticles. Thus, the influence of pH (4-8) on the synthesis of the silver nanoparticles was investigated. The results (Fig. 2c) showed that the intensity of absorbance increased with increasing pH from 4 to 5 and then decreased at higher pH (5-8). The increase in the absorbance indicated that the number of nanoparticles formed increased with increasing pH to 5; after that, the concentration of nanoparticles decreased. The peak shape (sharpness) and position changed insignificantly over pH 4 to 6, and broader peaks were observed at pH 7 & 8. The insignificant changes in the peak position and shape indicate the production of nanoparticles of similar sizes (monodisperse), and the peak broadness suggests that nanoparticles of polydisperse particle sizes were formed due to agglomerations (Liaqat *et al.,* 2022). The maximal peak absorbance was observed at pH 5, which was considered the optimal pH for the synthesis of nanoparticles in the following experiments.

The formation of nanoparticles involves a reduction of silver ions by using phytochemicals from plant extract. Consequently, the concentration of silver ions participating in the reaction affects the formation of nanoparticles. Thus, the influence of the concentration of silver ions (0.001 – 0.04 M) in the formation of nanoparticles was evaluated using a reaction time of 20 min and pH 5. The findings (Fig. 2d) showed that the yield of nanoparticles improved with increasing concentration of silver ions to 0.02 M and after that, a decreased concentration of the nanoparticles formed at 0.04 M. The increase in the concentration of nanoparticles at higher silver ion concentrations is a likely indicator that more silver ions are reduced. A similar increase in the concentration of silver nanoparticles formed with the concentration of silver ions was previously reported (Sobczak-Kupiec *et al.,* 2011). The observed

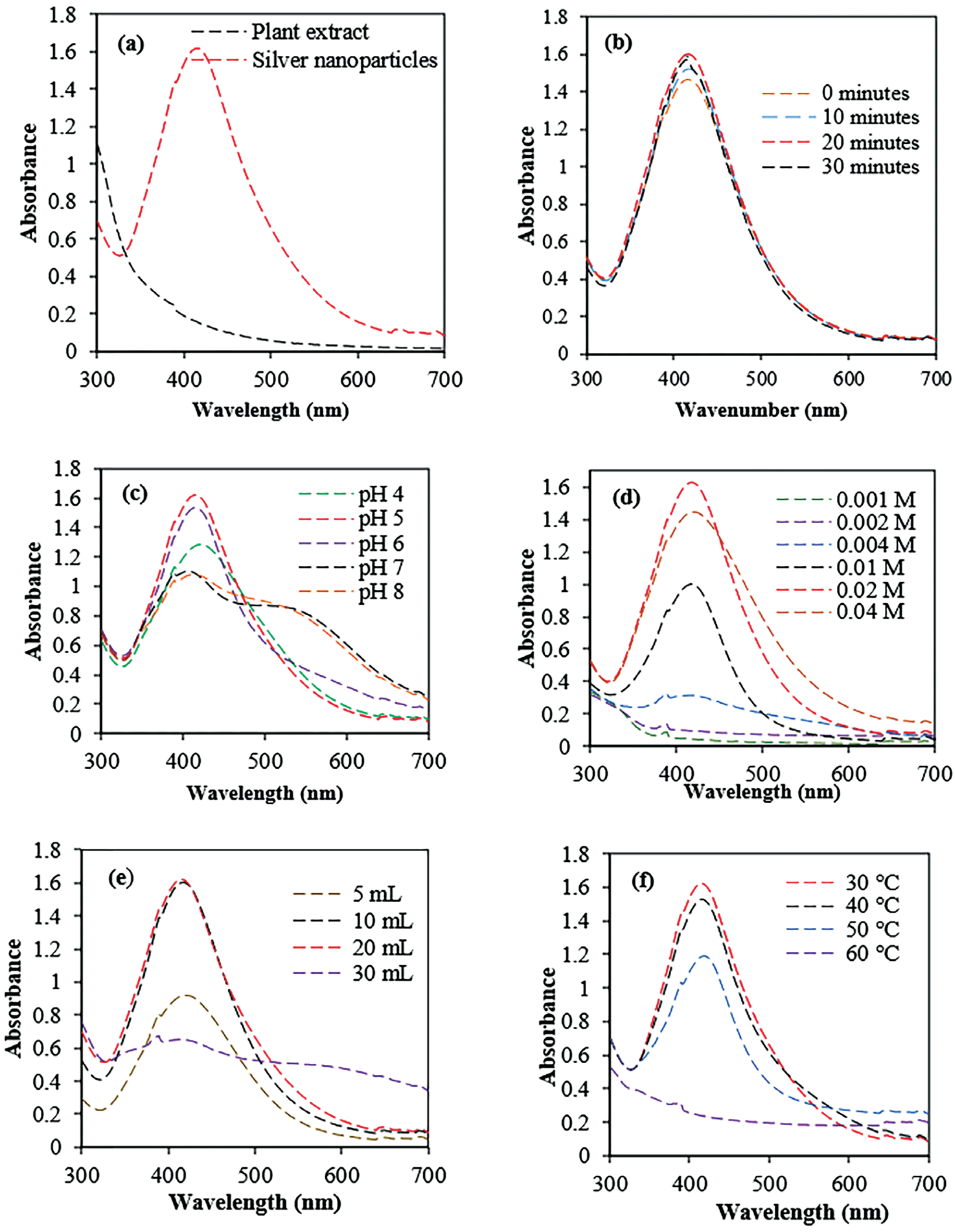

Figure 2: UV-Visible absorption spectra of ssynthesised ZPLAgNPs showing resonance peak at 420 nm (a) as a function of reaction time (b), pH of reaction (c), $AgNO_3$ concentration (d), extract volume (e), and reaction temperature (f).

decline in the absorption intensity at a higher concentration of $AgNO_3$ (0.04 M) was possibly a result of agglomeration due to Van der Waals forces between neighbouring nanoparticles (Reference). The findings in the current study showed that 0.02 M was the optimal $AgNO_3$ concentration required for the synthesis of silver nanoparticles at high yields without aggregations.

The amount of phytochemicals present

in the reaction mixture affects kinetics for the formation of the silver nanoparticles as they serve as reducing and stabilising agents (Kordy *et al.,* 2022; Pradeep *et al.,* 2022). The findings (Fig. 2e) indicated that absorbance increased with increased silver nanoparticles. Thereafter, a decrease in the absorbance was recorded at a higher extract volume (30 mL). The increase in the absorbance intensity with the extract volume indicates an increase in the phytochemicals involved in the reduction reaction. The decrease in the absorbance intensity at higher extract concentrations was attributed to agglomerations of silver nanoparticles. From the experiments, both 10 and 20 mL extracts were identified as optimal volumes for the synthesis of the nanoparticles. An extract volume of 10 mL was selected and used for subsequent experiments.

The influence of temperature (30–60 °C) on the formation of nanoparticles was also examined. The results (Fig. 2f) showed that the amount of silver nanoparticles produced decreased as the temperature was increased. Consequently, the lowest absorbance was observed at a higher temperature of 40°C. The position and shape of the absorbance peaks were similar over the temperature range of 30-50°C. The observation indicates that the particle size distribution was insignificantly affected by an increase in temperature from 30 to 50°C. However, the formation of silver nanoparticles was almost negligible at 60°C. This observation is a possible indicator of the decomposition of the phytochemicals in the aqueous extract before formation of silver nanoparticles. The observed trend contradicts other reported findings where the formation of silver nanoparticles increased with temperature (Liaqat *et al.,* 2022). The difference in the effects of temperature could be associated with differences in the volatility and stability of different phytochemicals. Therefore, 30°C was used as the optimal temperature for synthesising silver nanoparticles in the following experiments.

Antimicrobial properties of ZPLAgNPs

The findings (Table 2) indicated that the synthesised silver nanoparticles were effective against both gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*). MIC values showed that synthesised silver nanoparticles were more effective for gram-positive (0.07 mg/mL) than gram-negative (0.13 mg/mL). However, the minimum bactericidal concentrations (MBCs) indicated that gram-negative bacteria were killed at a lower concentration (0.13 mg/mL) compared to gram-positive bacteria (0.53mg/mL). Similarly, the silver nanoparticles displayed antifungal properties against Candida albicans and Aspergillus niger. The MBC values indicated that the nanoparticles were less effective against Candida albicans (1.06 mg/mL) than Aspergillus niger (0.13 mg/mL). The findings in this study demonstrate that the fabricated silver nanoparticles were effective against bacteria and fungi. Other silver nanoparticles have shown bioactivity against fungi and bacteria (Bernardo-Mazariegos *et al.,* 2019).

Table 2: Effectiveness of ZPLAgNPs against selected microorganisms

Micro-organism	MIC (mg/mL)s	MBC (mg/mL
Bacteria		
Escherichia coli	0.13	0.13
Staphylococcus aureus	0.07	0.53
Fungi		
Candida albicans	1.06	1.06
Aspergillus niger	0.13	0.53

The observed antimicrobial properties were attributed to their small sizes, allowing them to easily permeate a bacterial cell wall and generate Ag ions due to surface oxidation (Munir *et al.,* 2022). Silver ions produced after the nanoparticles permeate a microorganism cell wall may bind to DNA and cellular structural thiols. These can cause the alteration of DNA and affect the integrity of the cell wall. Silver ions can also bind with vital enzymes such as respiratory enzymes, making them inactivate. The ions can also interfere with ingesting phosphate ions and enhance their outflow from microorganism cells. Silver nanoparticles may also kill microbes by producing reactive oxygen species and free radicals that can damage the bacterial cell wall (Munir *et al.,* 2022). The preliminary findings of the present study suggest that the synthesised silver nanoparticles can serve as a potential antibiotic for treating bacterial and fungal diseases in humans and animals.

Conclusions

An aqueous extract from Zanthoxylum piperitum leaves was successfully prepared and used in the green chemistry synthesis of ZPLAgNPs using optimized conditions of reaction time 20 minutes, pH 5, ZPL extract volume 10 mL, AgNO3 concentration 0.002 M and temperature 30 °C. Analysis revealed that the plant extract contained phytochemicals such as tannins, phenols, flavonoids and saponins with reduction and stabilising properties. During the preparation of the silver nanoparticles using the aqueous extract, a significant colour change from light yellow to brown was noted, serving as a potential indicator for the formation of silver nanoparticles. UV-Vis spectrophotometry revealed the formation of a plasmon resonance band at 420 nm. The fabricated silver nanoparticles showed good antibacterial and antifungal properties, indicating their potential as alternatives to conventional antibiotics. However, more investigations on the characterisation of the nanoparticles are required to optimise their performance.

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