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One-step phyto-mediated fabrication of silver nanoparticles and its anti-microbial properties

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Abstract. This manuscript describes the one-step eco-friendly green fabrication of silver nanoparticles (AgNPs) through the in-situ bio-reduction of an aqueous solution of silver nitrate using Syzygium aromaticum leaf extract. UV–vis spectroscopy shows a characteristic SPR peak around 442 nm. FTIR spectroscopy showed that the AgNPs were capped with bioactive phyto-molecules. TEM images revealed oval and spherical particles with a mean diameter of ~12.6 nm. XRD analysis revealed crystalline and face-cantered cubic AgNPs. The phytosynthesized AgNPs showed broad-spectrum anti-microbial activity against two foodborne pathogenic bacteria, Listeria monocytogenes and Staphylococcus aureus. The AgNPs showed a prominent ability to inhibit biofilms formed by L. monocytogenes and S. aureus in laboratory conditions through a crystal violet assay. The results suggest that the AgNPs could be a novel nanotool to develop effective antimicrobial and anti-biofilm agents in food preservation.
Keywords: anti-bacterial; anti-biofilm; characterization; nanoparticles; phytosynthesis

1. Introduction

Nanotechnology allows us to develop new materials and methods to better understand nanomaterial interactions with intracellular structures, processes, and the environment. Nanotechnology creates pharmacological and biological nanoparticles (Morais *et al*. 2014). Most nanoparticle bio-fabrication research avoids hazardous solvents, reagents, wastes, and energy requirements by exploiting natural resources (Kumar *et al*. 2015). Researchers are studying spontaneous nanoparticle biosynthesis. Natural macro-molecules, rktmbacteria, yeast, fungi, plants, algae, photo-synthetic organisms, and marine animals biofabricate nanoparticles (Yosri *et al*. 2021). Cellular enzymes can also biofabricate. Microbial nitrate reductase turns metal ions into metallic nanoparticles in extracellular biofabrication. Product recovery from intracellular NP production is arduous and expensive (Iravani and Varma 2020). Fungus-secreted proteins and reducing agents keep extracellular nanoparticles stable (Mahendra Rai *et al*. 2009). Plant extract proteins, flavonoids, polyphenols, alkaloids, saponins, phenols, essential oils, and polyols bio-reduce metal ions to metallic nanoparticles and stabilize phyto-synthesized nanoparticles (Barabadi *et al*. 2020, Taha 2022). Metallic nanoparticles can non-destructively attach to single-stranded DNA for medical diagnostic and therapeutic purposes (Tremi *et al*. 2021). Hybrid polymer/ protein conjugation with nanoparticles transfers undamaged DNA to cell nuclei, increasing gene expression. Nanoparticle bio fabrication employing microorganism-secreted enzymes is a rational, straightforward, and eco-friendly large-scale bioprocess (Barabadi 2017).

Silver nanoparticles (AgNPs) have received attention due to their extensive applications in areas such as anti-microbials, optics, catalysis, and biomaterial production (Burduşel *et al*. 2018). Nanoparticles have been effectively used in various fields, like in medicine as an antibacterial for skin and burn wounds, and wound dressings, as well as functionalized fabric, medical devices to avoid bacterial contamination, and food processing (Supraja *et al*. 2017, 2018). Various living organisms such as fungi, bacteria, algae, actinomycetes, and plants have been employed for the fabrication of nanoparticles with an augmented anti-microbial ability (Honary *et al*. 2013, Varadharaj *et al*. 2020, Chugh *et al*. 2021, Truong *et al*. 2022). This feature is important for effective biomedical applications (Gupta *et al*. 2014, Singh *et al*. 2018) of nanoparticles. Silver has long been acknowledged as a valuable metal with numerous commercial advantages in addition to its medicinal advantages, such as its antibacterial and anti-diabetic properties. Concern has been expressed about the possibility that herbal-mediated nanoparticles' physical and chemical

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characteristics may interact with the biological system in novel, surprising ways (Shanker *et al*. 2017). In addition, the pharmaceutical and biological potential of green synthesized silver nanoparticles such as antiviral (Barabadi *et al*. 2022a), antibacterial (Barabadi *et al*. 2021), antifungal (Barabadi *et al*. 2022a), antiparasitic (Gaafar *et al*. 2014), antioxidant, anticoagulant (Talank *et al*. 2022), and biofilm inhibitory (Barabadi *et al*. 2022b) activities of bio-synthesized silver nanoparticles and also the anticancer activity of biosynthesized silver nanoparticles against different cancers such as lung (Barabadi *et al*. 2020a), colorectal (Barabadi *et al*. 2020b), leukemia (Mostafavi *et al*. 2022), hepatic (Barabadi *et al*. 2020b), etc.

The investigation of plants for the synthesis of nanoparticles has benefits over physical, chemical, and microbial syntheses since they do not require additional processes of culturing and preserving the cells, hazardous chemicals, high energy, or wasteful purifications (Das *et al*. 2017, Sharma *et al*. 2019). The leaves, cloves (one of the most valuable spices), and clove oil of *Syzygium aromaticum* have been traditionally used in folk medicine, food flavoring, food preservatives, fragrances, pharmaceuticals, and antibacterial, antifungal, and antiseptic agents. In a food factory environment, several bacterial pathogens form biofilms on the artificial substrates common in the food industry (Guidelli *et al*. 2011, Kouvaris *et al*. 2012, Sulaiman *et al*. 2013). Furthermore, the antimicrobial resistance developed by pathogenic bacteria is a major issue. To overcome this, there is a necessity to develop silver-based nanoparticles with broad-spectrum activity to lower microbial resistance to antibiotics (Abdel-Aziz *et al*. 2014).

In this study, we developed an environmentally friendly and economical route for the fabrication of AgNPs with *S. aromaticum* leaf extract. The synthesized AgNPs showed anti-microbial properties against *Listeria monocytogenes* and *Staphylococcus aureus*. These NPs might open a new avenue and have potential use in the biomedical field. A green and environmentally friendly method for the synthesis of silver nanoparticles using aqueous extracts was developed in this study, and the one-step phyto-mediated fabrication of silver nanoparticles, physical characterization, and its anti-microbial properties using *S. aromaticum* leaf extract is a novel finding. Therefore, the research may shed light on a novel route that could one day replace the need for antibiotics.

2. Experimental section

For the reduction of Ag^+ ions, 10 ml of *S. aromaticum* leaf extract was added to 90 ml of 1×10^{-3} M aqueous $AgNO_3$ solution in a 250-ml Erlenmeyer flask. The initial production was confirmed by the visual appearance of the color change from light yellow to dark brown. The optimum conditions of AgNP production were identified by varying production parameters like the pH (3-10), substrate concentration (0.5-1.5 ml), and metal ion concentration (0.05-0.5 mM). We periodically observed the reaction mixture (extract + Ag^+ ion) with wavelengths of 200-800 nm using UV–vis spectroscopy (Multiscan spectro-photometer, Thermo Scientific) (Hirsch *et al*. 2005, Velmurugan *et al*. 2015, 2016)

The AgNPs obtained from the reaction mixture were refined by repeated centrifugation at 12,000 rpm for 20 min and lyophilized to obtain AgNP powder. This was followed by the dispersion of the pellet in deionized water to remove biomolecules present in the synthesized product arising from the extract and metal ions (Prabhu *et al*. 2013, Shetty *et al*. 2014, Sharma *et al*. 2018). The physical characterization and morphology of the attained powder were achieved using a scanning electron microscope and energy-dispersive X-ray spectroscopy (SEM/EDS, JEOL Moel, JSM 6390LV, Japan). The crystalline nature was confirmed by an X-ray diffractometer with CuKα radiation (XRD, Rigaku, Miniflex), and Fourier transforms infrared spectroscopy (FT-IR, Perkin-Elmer, Spectrum 100) was used to identify the possible bioactive functional groups present in the AgNPs as a capping and stabilization agent (Prasad *et al*. 2011).

The morphology and size of AgNPs were identified using HR-TEM (FEI Technai G2 F205-TWIN TEM) by placing a few drops of sonicated samples on a carbon-coated copper grid placed on filter paper on a hot plate. The antibacterial activity was studied using food-born pathogenic bacterial strains (*L. monocytogenes* (ATCC 19111) and *S. aureus* (ATCC 13565)) with the agar-well diffusion method. Briefly, mid-log cultures grown on BHI broth (100 µl (10^5 CFU/ ml)) Colony formation units (CFU) were swabbed on an MHA plate containing wells of uniform size. The wells were loaded with AgNPs (5-40 mg/ml), aqueous extract (5-40 µl/ml), AgNPs with extract (5-40 mg/ml), and standard antibiotics (Amoxicillin). The plates were incubated at 37°C for 24 h and evaluated for antimicrobial activity by measuring the zone of inhibition (mm).

A biofilm formation and inhibition assay was performed in 96-well microliter plates according to Liu *et al*. (Liu *et al*. 2019). Briefly, *L. monocytogenes* and *S. aureus* were grown in BHI, and the cells were harvested by centrifugation. This was followed by re-suspending the cells in saline at approximately 1×10^8 CFU/mL. Next, 0.1 ml of cultures were mixed with different concentrations of AgNPs (2 to 256 mg/ml), extract (10 to 100 µl), and AgNPs with extract (2 to 256 mg/ml), which were added to four separate wells in the 96-well microplate for replicate testing.

After 48 h of incubation, the wells were decanted and washed with 1X PBS buffer, dried, stained with 200 µL of 0.1% (w/v) crystal violet (CV), and retained for 15 min at ambient temperature. Later, the CV was removed, and wells were rinsed with PBS, followed by the addition of 200 µL of 95% (v/v) ethanol. Biofilm quantification was done by measuring the optical density at 590 nm with appropriate control. The reduction of the biofilm was calculated in the presence and absence of AgNPs in wells.

3. Results and discussion

The UV–vis absorption spectrum of AgNPs shows a

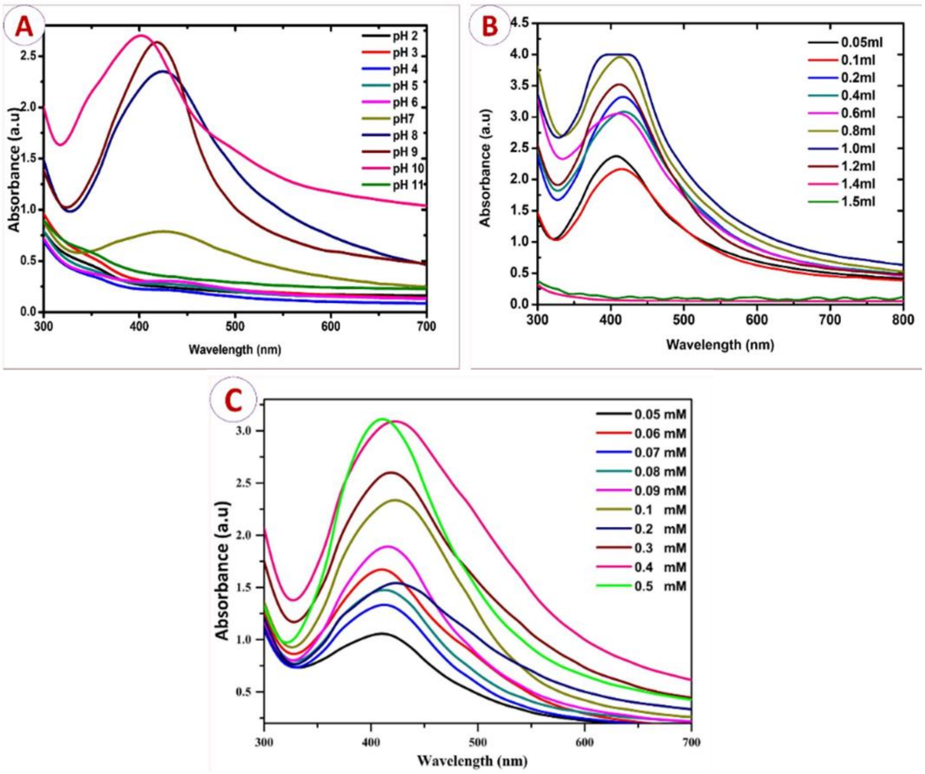

Fig. 1 (a)-(c) UV–vis spectra of AgNPs using *S. aromaticum* leaf extract as a function of (a) pH, (b) extract and (c) silver ion concentration indicated by an absorbance peak at 442 nm

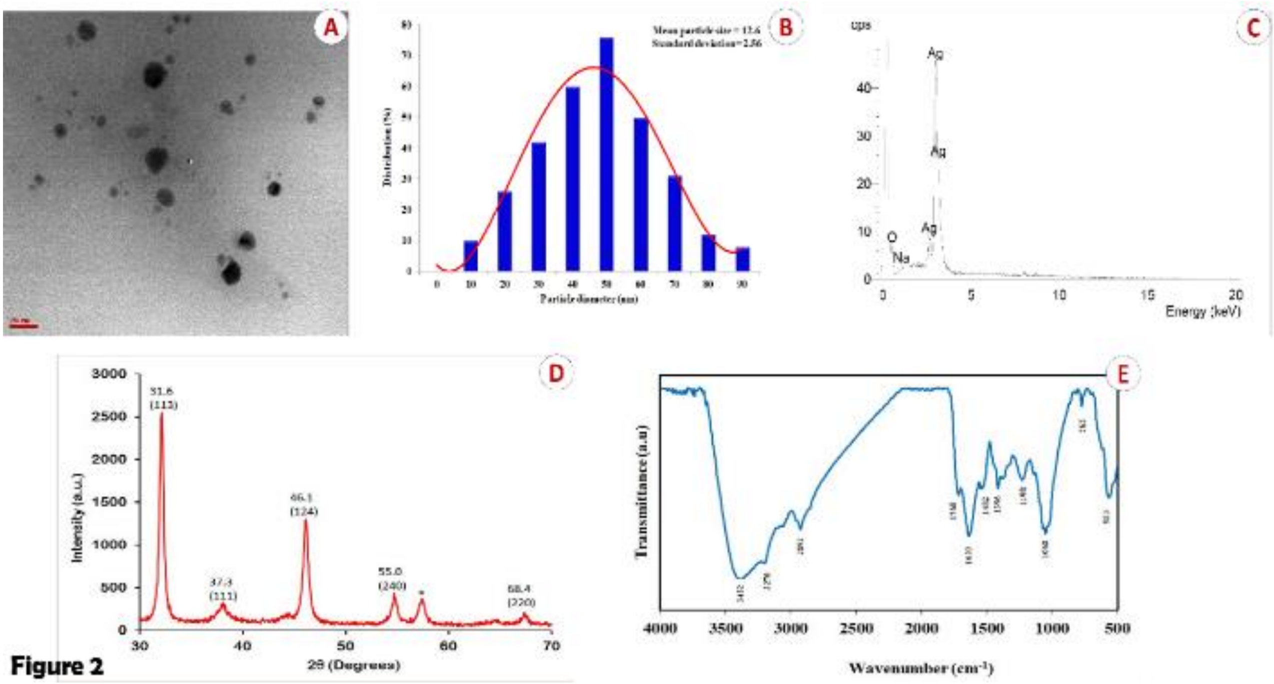

Fig. 2 (a)-(e) (a) TEM micrographs, (b) histogram, (c) EDS spectrum, (d) XRD, and (e) FTIR of phytofabricated AgNPs.

rapid color change as an indication of AgNP formation at ambient temperature, as shown in Fig. 1 for different conditions. The optimal conditions were pH 10 (Fig. 1a), 0.8 ml of extract (Fig. 1b), and a metal ion concentration of 0.5 mM (Fig. 1c). There was a broad surface plasmon resonance (SPR) peak at 442 nm (Supraja *et al*. 2018, Baláž *et al*. 2019). A similar result was reported by Gomathi *et al*. 2020 for the synthesis of AgNPs using *Gymnema sylvestre* leaf extract, which showed an SPR peak at 442 nm. The short and long wavelengths and size of the green-synthesized

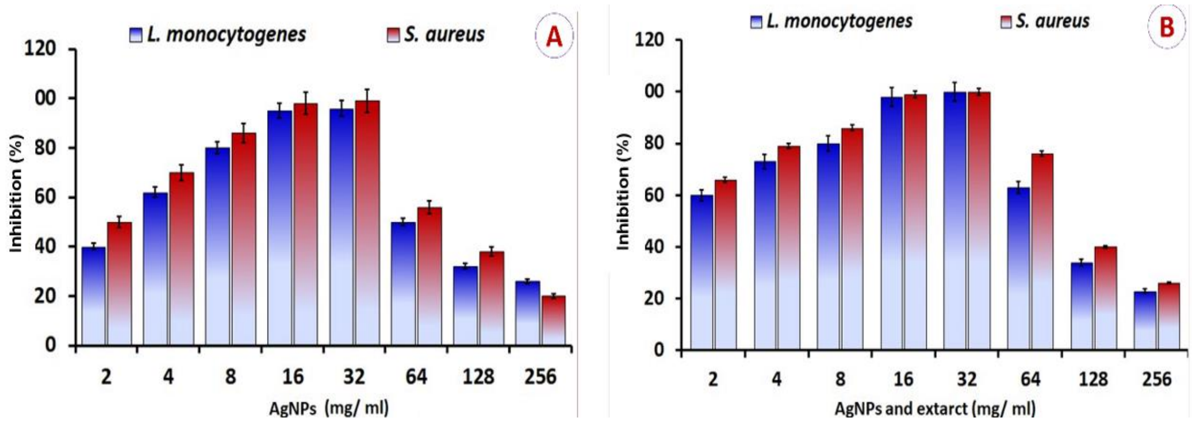

Fig. 3 Anti-biofilm activity of AgNPs (2-256 mg/ml) on (a) *AgNPs*, and (c) *AgNPs with extract* on a 96- well plate using crystal violet assay

Table 1 Effect of AgNPs, extract and AgNPs with extract on food-born bacteria *L. monocytogenes* and *S. aureus*

Food born bacteria	The concentration of AgNPs (mg/ml)			
	5 mg	10 mg	20 mg	40 mg
	Zone of inhibition (mm)			
L. monocytegenes	3±0.2	5±0.6	7±0.2	9±0.4
S. aureus	3±1.0	5±0.3	7±0.8	9±0.6
The concentration of extract alone (mg/ml)				
L. monocytegenes	-	-	-	2±10.2
S. aureus	-	-	-	2±0.6
The concentration of AgNPs with extract (mg/ml)				
L. monocytegenes	3±0.2	6±0.4	9±0.7	10±1.6
S. aureus	3±0.9	5±0.2	7±0.4	9±1.3

AgNPs are due to the difference in pH of the reaction mixture (Sivakumar *et al*. 2012, Traiwatcharanon *et al*. 2017).

The HR-TEM image (Fig. 2a) shows the morphology and the size of the obtained AgNPs, which are mostly dispersed in spherical and oval-shaped particles with an average particle size of ~20 nm (Fig. 2b). The EDS spectrum shows a strong silver signal along with weak oxygen, sodium, and carbon peaks, which may originate from the carbon tape and biomolecules that are bound to the surface of the AgNPs (Fig. 2c). The total phenolic content present in the leaf extract was higher than in the AgNPs (31±1.3 and 0.9±1.8 GAE/100 mg), which were analyzed using gallic acid (GA) as a positive control.

Fig. 2d shows the crystalline nature of the synthesized AgNPs, which shows good accordance between the detected experimental diffraction angle [2θ] and the standard diffraction angle [2θ]. The diffraction patterns of AgNPs can be indexed to face-cantered-cubic silver (JCPDS file no. 04-0783) (Gupta *et al*. 2014; Velmurugan *et al*. 2015, 2016). The diffraction peaks at 2θ values of 31.60°, 37.30°, 46.10°, 55.00 and 68.40° can be attributed to the reflections of the (113), (111), (124), (240), and (220) planes of face-cantered cubic silver, respectively.

The bioactive molecules responsible for the reduction and formation of AgNPs were examined by FT-IR (Fig. 2e). Eugenol, β-caryophyllene, beta-caryophyllene, α-humulene, α-farnesene, and caryophyllene oxide are the key chemical constituents of *S. aromaticum* leaf extract (Gupta *et al*. 2014). Several earlier reports revealed that phenols and related bioactive phytoconstituents are the key sources of reducing, stabilizing, and antimicrobial components in AgNPs (Dahl *et al*. 2007, Dash *et al*. 2014).

Table 1 shows the antibacterial activity of AgNPs, extract, and AgNPs with the extract. As evident from the clear zone of inhibition, moderate activity was observed in AgNPs and AgNPs with extract, but there was no activity in extract alone (Salem *et al*. 2014, Shanmugam *et al*. 2014). The AgNPs obtained from clove oil and clove broth show good antibacterial activity against the studied bacteria (Kaur *et al*. 2013, Gao *et al*. 2017), which is due to the presence of several antibacterial compounds in clove. However, our results on the antibacterial and antibiofilm efficiency showed better activity than earlier reports (Loo *et al*. 2018).

AgNPs showed effective anti-biofilm activity at very low concentrations (16 and 32 mg/ml), and higher concentrations (64-256 mg/ml) lead to less antibiofilm activity, which might due to the aggregation of AgNPs around the cells (Figs. 3a and b). However, the control (extract) showed no anti-biofilm activity. The antibacterial action of AgNPs is due to the diffusion into the cell and cell surface, contact with proteins, restriction of the replication of cells, and the arrest of cell metabolism (Gupta *et al*. 2014; Velmurugan *et al*. 2015, 2016). Additional factors that may influence the biological activity of inorganic nanoparticles like size distribution, morphology, surface charge, surface chemistry, capping agents, etc (Javed *et al*. 2020, Barabadi *et al*. 2020c, d, Saravanan *et al*. 2021a, b). Multiple metal NPs have been reported to enter cells and harm their membranes, DNA, and proteins. Also, because of their diminutive size, NPs can circulate throughout the body and eventually accumulate in the brain, heart, and lungs, where they can cause significant toxicity (Ferdous and Nemmar 2020, Attarilar *et al*. 2020).

4. Conclusions

We effectively developed a low-cost, fast, and one-pot procedure for the phyto-fabrication of AgNPs using clove tree leaf biomass. The results confirmed the in-situ bio-reduction of Ag^{+} ions. AgNPs showed effective anti-microbial behavior against two food-borne pathogenic bacteria at a higher concentration of AgNPs and AgNPs with the extract. In conclusion, the phytofabricated AgNPs could have applications in antibacterial and antibiofilm coating materials for machines in the food industry, tanks, pipelines, centrifuges, and packing tools.

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