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Multifunctional biosynthesized silver nanoparticles exhibiting excellent antimicrobial potential against multi-drug resistant microbes along with remarkable anticancerous properties

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ABSTRACT

This study demonstrates the therapeutic potential of silver nanoparticles (AgNPs), which were biosynthesized using the extracts of *Citrus maxima* plant. Characterization through UV–Vis spectrophotometry, Dynamic Light Scattering (DLS), Fourier Transform Infrared spectroscopy (FTIR), X-ray Diffraction (XRD) and Transmission Electron Microscopy (TEM) confirmed the formation of AgNps in nano-size range. These nanoparticles exhibited enhanced antioxidative activity and showed commendable antimicrobial activity against wide range of microbes including multi-drug resistant bacteria that were later confirmed by TEM. These particles exhibited minimal toxicity when cytotoxicity study was performed on normal human lung fibroblast cell line as well as human red blood cells. It was quite noteworthy that these particles showed remarkable cytotoxicity on human fibrosarcoma and mouse melanoma cell line (B16-F10). Additionally, the apoptotic topographies of B16-F10 cells treated with AgNps were confirmed by using acridine orange and ethidium bromide dual dye staining, caspase-3 assay, DNA fragmentation assay followed by cell cycle analysis using fluorescence-activated cell sorting. Taken together, these results advocate promising potential of the biosynthesized AgNps for their use in therapeutic applications.

1. Introduction

Nanotechnology is a multidisciplinary area that deals with synthesis, designing and manipulation of particles at nano-size range. These nanoparticles exhibit unique physicochemical, mechanical, electronic, catalytic, optical and thermal properties due to increased surface area-to-volume ratio, which are not present in the bulk form of materials [1,2]. Thus, nanoparticles have triumphed substantial deliberations in various fields such as drug and nucleic acids delivery, catalysis, single electron transistor, food, cosmetics, health, chemical industries, and biomedical sciences [3]. Amid numerous nanoparticles, silver nanoparticles (AgNps) have extensively been used in the various fields including medicine and biology [4,5]. Physical methods produce low yields of the particles and chemical methodologies are toxic, expensive and eco-unfriendly [6,7]. Green strategies utilizing natural capping, reducing and stabilizing agents to synthesize nanoparticles with craved morphology and size is a noteworthy angle for scientists. These methods include the utilization of bacteria, algae, fungi and plants [8–10]. However, synthesis, using plants, affords a better platform as they offer natural and non-toxic capping and stabilizing agents along with minimizing the expenditure of microorganism's isolation. The synthesis of nanoparticles using plants is less time consuming, biocompatible, fetches high yield and the size of nanoparticles can be managed by controlling parameters such as temperature, concentration of the reactants, speed of stirring and pH [11].

Emergence of multi-drug resistant pathogenic microorganisms demands implementation of novel antimicrobials [12]. Methicillin-resistant *Staphylococcus aureus*, multi-drug resistant (MDR) *Pseudomonas aeruginosa* and *Salmonella enteritidis* are known for their drug dodging mechanisms [13,14]. Not only these, but some bacteria residing on the human skin such as *Propionibacterium acnes*, a skin commensal, turns pathogenic in the case of acne vulgaris, developing resistance against antibiotics like tetracycline, minocycline, clindamycin, etc., hence challenging scientists to develop novel and effective drugs [15,16]. Thus, it becomes mandatory to come up with strategies that can facilitate pivotal bacterial killing. Traditional antimicrobial approaches suggest silver as an antimicrobial agent, thus its potential need to be explored [17,18]. Along with these, there are several reports to support the efficient

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antimicrobial potential of silver nanoparticles having rare chance for the development of drug-resistance [19–25]. Along with antimicrobial potential, these nanoparticles have also shown to impart their role as potential anticancerous agents [26–29].

In the present study, extracts from various parts of *Citrus maxima* plant were used as novel and green source of precursors in order to biosynthesize the silver nanoparticles (AgNps). These particles showed enhanced antioxidant activity, excellent antimicrobial as well as anticancerous activity without much affecting the normal human cell lines and RBCs. Taken together, these biosynthesized silver nanoparticles might serve as drugs next door for various outrageous diseases including cancer.

2. Materials and methods

2.1. Biosynthesis of silver nanoparticles using C. maxima extracts

The plant extract preparation was performed, as described previously [30,31]. Briefly, healthy fruit, leaves and peel were collected and cleaned with autoclaved Milli Q water. Fruit pulp was crushed and juice was strained through Whatman filter paper No. 1 (HiMedia) followed by centrifugation at 12000 rpm for 45 min at 4 °C and then filtered using 0.2 μm membrane (HiMedia). Whereas, dried leaves and peel were crushed in a mixer. Fine leaf and peel powder (~10 g) were suspended in separate vessels in 100 ml of Milli Q water and heated in an oil bath at 90 °C for a period of 3–5 h. Then, these were cooled, filtered, lyophilized and stored at 4 °C. In order to biosynthesize silver nanoparticles, fruit juice (10 ml) was added dropwise in a flask containing 200 ml of 1 mM $AgNO_3$ solution with constant stirring at 1000 rpm at room temperature. For leaf and peel extracts too, 10 ml of the extract was added to other flasks containing 200 ml of 1 mM $AgNO_3$ solutions, respectively. Color of the solution changed from colorless to brownish black within 4–6 h. It was centrifuged at 12000 rpm for 45 min to obtain the AgNps pellets, followed by its extensive washings with water and lyophilization [32]. In addition, chemically synthesized silver nanoparticles were also prepared by using sodium borohydride ($NaBH_4$) (Sigma), which were used as a control.

2.2. Characterization of synthesized nanoparticles

Synthesis of AgNps was confirmed by recording their spectra (200–850 nm) using UV–Vis spectrophotometer. Aqueous solutions of synthesized AgNps (1 mg/ml) were used for Dynamic Light Scattering (DLS) measurement using Zetasizer Nano-ZS (Malvern Inc., UK). Typically, AgNps (1 mg) were dispersed in 1 ml of Milli Q water by sonication (3 × 5 min). After formation of homogeneous solution, size and zeta potential measurements were performed. Average values of these parameters were obtained in automatic mode for 20 and 30 runs, respectively. Fourier Transform Infrared (FTIR) spectra of these nanoparticles were recorded using a single-beam Spectrum RXI-MID-IR (PerkinElmer, USA), with the following scan parameters: scan range, 4400–400 cm^{-1}; number of scans, 16; resolution, 4 cm^{-1}; interval, 1 cm^{-1}; unit %T. Lyophilized samples of synthesized AgNps were subjected to X-ray diffraction (XRD) measurements with an X'pert PRO XRD (Analytical BV, Almelo, Netherlands), operating in transmission mode at 30 kV, 20 mA with Cu-Ka radiation [32]. The size and shape of AgNps were analyzed using Transmission Electron Microscopy (TEM), as described previously [33]. The TEM micrographs were taken at an accelerating voltage of 200 kV (Tecnai G2 30 U-twin, 300 kV Ultra-twin microscope).

2.3. Antioxidant activity

The antioxidant activity of synthesized AgNps was performed following a previously reported method [34]. Briefly, the dilutions of *C. maxima* extract as well as biosynthesized silver nanoparticles were made in the range of 50 to 1000 μg/ml. 100 μl of each diluted samples was mixed with 100 μl of 2,2-diphenyl-1-picrylhydrazyl solution (80 μg/ml, DPPH, HiMedia) in a 96-well plate (Corning) and the final volume was made up to 200 μl. The plate was incubated in dark for 30 min at room temperature. Quercetin and methanol were used as positive and negative controls, respectively. Finally, the DPPH free-radical scavenging activity (%) was determined at 517 nm using Tecan microplate reader.

2.4. Antimicrobial activity

The antimicrobial activity of these synthesized AgNps was performed against various pathogenic microorganisms such as *Escherichia coli* MG1655 (MTCC 1586), *Bacillus cereus* (MTCC 430), *Bacillus subtilis* (MTCC 121), *Klebsiella pneumoniae* (MTCC 3384), *Pseudomonas aeruginosa* (MTCC 741), *Staphylococcus aureus* (MTCC 740), multi-drug resistant *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13076), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300). The antimicrobial activity was also performed against several clinically isolated pathogenic microorganisms from acne lesions, viz., *Klebsiella pneumoniae* (MCC 2748), *Acinetobacter radioresistens* (KF 954714), *Enterobacter xiangfanfensis* (MCC 2770), *Propionibacterium acnes* (KF 268368) and *Staphylococcus haemolyticus* (KF 268371). All the multi-drug resistant (MDR) strains and acne pathogens were cultured in Brain Heart Infusion media (HiMedia) at 37 °C. Whereas, others microorganisms were maintained in Luria Bertani media (HiMedia) at 37 °C. Gentamicin (0.01 mg/ml) (HiMedia) and autoclaved Milli Q water were used as positive and negative controls, respectively. Inoculum of optical density 0.5 (10^5 colony forming units/ml) was prepared by taking pure colony with a sterile inoculating loop from agar plate in sterile broth, incubated overnight at 37 °C.

The antimicrobial activity was performed by using Kirby-Bauer disk diffusion assay as described previously [35,36] Sterile disks of 6 mm, impregnated with the synthesized AgNps (100 μg/disk), *C. maxima* extracts (1 mg/ml), $AgNO_3$ (1 M), Gentamicin (positive control) and sterile Milli Q water (negative control), were placed on Mueller Hinton agar plates seeded with test microorganism followed by an overnight incubation at 37 °C. The appeared zone of inhibition (mm) around each disc was measured using HiAntibiotic zone scale (HiMedia). To analyze the minimum inhibitory concentration of AgNps against these microbes, microbroth dilution assay was performed in 96-well plates [33]. Serial dilutions of AgNps (1 to 0.0005 mg/ml) with 10 μl of mid-log phase bacterial culture and Mueller Hinton Broth (HiMedia) were finally made to 200 μl. Wells with only media and bacterial culture served as growth controls. Absorbance was measured by using TECAN spectrophotometer at 600 nm, before and after overnight incubation at 37 °C. Experiment was done in triplicates. After overnight incubation, 50 μl of 200 μg/ml of *p*-iodonitrotetrazolium chloride (HiMedia) was added to the each well and further incubated at 37 °C for 30 min to monitor the bacterial growth.

2.5. Transmission Electron Microscopy (TEM)

Transmission electron microscopic analysis of AgNps treated pathogenic microorganisms was performed as carried out previously [33–36]. Briefly, fresh culture of *B. cereus* and *S. enteritidis* were treated with AgFeNps, AgLeNps and AgPeNps (1 mg/ml) for 30 min at 37 °C and then centrifuged at 4000 rpm for 10 min. The untreated bacteria served as control. The pellet, so obtained, was washed with 1X PBS (pH 7.4) and then fixed with 4% paraformaldehyde (Sigma) at 4 °C overnight. Later, the dehydration of samples was done by washing with gradually increasing concentration of ethanol (Merck) (10–100%). Bacterial pellets were resuspended in 200 μl of autoclaved Milli Q water followed by staining with 1% uranyl acetate (HiMedia) for 5 min. 10 μl of the stained bacterial cells were loaded on carbon-coated TEM grids (200 mesh Cu, TED Pella Inc., USA), air dried and TEM micrographs were taken.