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ROS mediated destruction of cell membrane, growth and biofilms of human bacterial pathogens by stable metallic AgNPs functionalized from bell pepper extract and quercetin

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ABSTRACT

Yellow pepper extract and quercetin (QDH) were used for YPE-AgNPs and Q-AgNPs fabrication. The AgNPs were thoroughly characterized using standard physico-chemical techniques and were found monodispersed, pleomorphic and had variable shape and size with a lattice fringe of 0.23 nm. YPE-AgNPs and Q-AgNPs revealed a characteristic SPR band at 438 nm and 431 nm. The XRD crystal size of YPE-AgNPs and Q-AgNPs was 10.16 and 12.20 nm while TEM analysis showed a size range of 5–40 and 1–25 nm. Bio-fabricated AgNPs remained stable for at least four weeks as the SPR did not deviate with time. FTIR data revealed functionalization of AgNPs by organics of reaction mixture. AgNPs had robust antibacterial and antibiofilm activity against ESβL(+) *Escherichia coli*, *Pseudomonas aeruginosa*, and methicillin sensitive and resistant *Staphylococcus aureus*. Staining of isolates with fluorescent probes displayed the increased production of ROS and membrane permeability. AgNPs hampered EPS production, endorsed DNA leakage, and generated superoxide radicals. Time and concentration dependent experiments demonstrated a consistent decrease in bacterial growth. Structural changes viz. irregular margins, distortion, depressions/indentations and shrinkage of cells were obvious under SEM. AgNPs due to strong antibacterial activity could be exploited as a supplement with antibacterial drugs to control resilient human infections.

1. Introduction

The constantly increasing use of nanoparticles in biosensor development, medical diagnosis, pharmaceutical and therapeutics is gaining a great momentum [1,2]. And hence, the synthesis of novel nanoparticles with fixed shape and size has been widely used to develop some novel antibacterial drugs for effective management of both plants and human bacterial pathogens [3]. The control over size and shape of nanoparticles alters their optical, chemical and biological traits [4]. Also, the greater surface area to volume ratio of nanoparticles relative to their bulk counter parts, their ability of absorption in visible region and reduction in particle size increases their therapeutic value against a range of diseases with minimal side effects [5,6]. Considering the importance of nanoparticles, several physical, chemical and biological methods have been developed and adopted to synthesize metal nanoparticles [7,8]. Of these, chemical methods which employ reducing agents such as sodium borohydrate have been found expensive and highly toxic [9]. Due to these, there is an urgent need to find an inexpensive and environmentally friendly strategy to produce safe, inexpensive and effective therapeutic nanoparticles. In this regard, plant extracts and phenolic compounds purified from plants have been found as a valuable alternative to hazardous physico-chemical methods used for nanoparticles generation [10]. The eco-friendly green synthesis involves phytochemicals for biomolecular reduction with better control over shape and size without showing any toxicity [11]. Plant and plant derived compounds mediated synthesis of nanoparticles offer certain important advantages including- (i) it is cost effective and eco-friendly (ii) method can be easily scaled up for pilot scale production and (iii) it does not require high energy, pressure, temperature and use of toxic chemicals. Among plant metabolites, flavonoids, terpenoids, proteins, reducing sugars and phenolic acids have been found to play key roles in the reduction of metal ions leading to the formation and stabilization of nanoparticles [12,13].

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Infectious diseases caused by pathogenic bacteria are major health concern worldwide, affecting large fraction of human populations [14]. On the contrary, the continuous increase in emergence of resistance to many drugs among pathogenic microorganisms is a matter of serious concern [15]. Therefore, there is an urgent need to develop novel antimicrobial agents or nano-antibiotics which could help to combat microbial infections inexpensively. Of these, metallic nanoparticles possess significant antimicrobial properties against multiple antibiotic resistant microbial species and are being considered as new generation of antimicrobials [16]. Among nano-particles, silver nanoparticles (AgNPs) in particular, have received greater attention as nano-antibiotics [9,17] due to their unique catalytic activity and greater chemical stability [18]. Also, AgNPs show better antimicrobial activity than other metal nanoparticles. Moreover, silver in nano form causes little destruction to mammalian cells [19]. Due to these, the AgNPs are considered as a powerful weapon against multidrug-resistant (MDR) pathogenic bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* [20].

Coloured bell pepper *(Capsicum annuum*) extract contains various active biomolecules such as proteins, enzymes, polysaccharides, amino acids, flavonoids and vitamins which act as reducing agent in extracellular synthesis of AgNPs [21]. Recently, numerous workers have used the *Capsicum* extracts for green synthesis of various kinds of nanostructures which varied in size from 10 to 40 nm [22,23]. As an example, silver-based nanostructures (metallic AgNPs and silver organometallic nano-disks) were prepared through reduction/oxidation process of $AgNO_3$ solutions mediated by fruit extracts of *C. annuum* var. aviculare at room temperature [24]. Similarly, other phyto-compounds such as flavonoids have shown many activities such as antioxidant, anticancer, antiviral, anti-allergic, cardiovascular protection potentials, and anti-inflammatory. Due to these, they are used regularly to improve human health [25,26]. Quercetin among flavonoids has free radical scavenging activity and is a very strong chelating agent. It is also known to have protective roles against cancer and heart diseases and help to regulate insulin and blood sugar level in diabetics [27]. Furthermore, quercetin alone and in combination with gallic acid has been used for the synthesis of bimetallic silver-selenium (Ag-Se) nanoparticles [8]. Bell pepper extracts are known to contain quercetin in highest amount as compared to other flavonoids and it is sufficiently thermally stable up to 180 °C without any adverse effect on its reducing potential [28,29].

Considering the importance of bell pepper, the present study was designed to achieve the following objectives- (i) synthesis of AgNPs using aqueous extract of yellow bell pepper (ii) pure phenolic compound QDH (iii) stability determination of synthesized AgNPs under different reaction medium (iv) assessment of antibacterial potential of AgNPs, biofilm inhibition ability, reduced production extracellular polymeric substance (EPS), reactive oxygen species (ROS) and superoxide radicals (O_2^-) generation (v) membrane permeability, eDNA release determination, evaluation of nanoparticles impact on ESβL (+) *E. coli*, *P. aeruginosa* and methicillin sensitive and resistant *S. aureus* under SEM equipped with EDX, and (vi) elucidation of mechanism of AgNPs formation, their interaction with bacteria and destruction of bacterial cells.

2. Materials and methods

2.1. Bacterial strains, plant materials and chemicals

Bacterial strains of human pathogens were same as recently used elsewhere [30]. Clinical isolates of extended spectrum beta lactamases ESβL (β) *P. aeruginosa* and *E. coli* and methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) were obtained from the culture collection of Jawaharlal Nehru Medical College & Hospital (JNMCH), Aligarh Muslim University (AMU), Aligarh (27°53′N 78°05′E 27.88°N 78.08°E), Uttar Pradesh, India. The potent ESβL producers were revalidated in our laboratory using Kit III for ESBL identification (SD240) procured from Hi-Media, India. Bacterial isolates were sub-cultured in Luria broth (LB) and Brain Heart Infusion (BHI) broth and maintained on LA and BHI agar slants (1.8%) at 4 °C and were sub-cultured regularly. Fresh yellow bell peppers were purchased from local market. Silver nitrate ($AgNO_3$, purity 99%) used for the synthesis of AgNPs and 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) ≥97% purity was purchased from Sigma-Aldrich. Acetone was purchased from Super Religare Laboratory (SRL), Mumbai, India. QDH ($C_{15}H_{10}O_7{\cdot}2H_2O$, assay 98%) and Propidium iodide (PI) and other chemicals were procured from HiMedia, Mumbai, India. Double distilled water (DDW) was used throughout the experiments and all reagents were of analytical grade.

2.2. Preparation of yellow bell pepper extract and QDH solution

Yellow bell pepper purchased from the local market of Aligarh, India was thoroughly washed several times with tap water followed by DDW and deionised water to remove the seeds, particulate matters and other surface contaminants. The washed fruits were chopped into small pieces and 25 g of chopped material was suspended in 100 ml DDW. The YPE was prepared following hot water extraction method, and kept at 100 °C for 20 min. The extract was then cooled and filtered using Whatman no. 1 filter followed by centrifugation at 8000 rpm to remove small solid particles. The supernatant was collected and stored at 4 °C until use. Various molar concentrations (25, 50, 75, and 100 μM) of QDH (CAS No. 6151–25-3; HiMedia, Mumbai, India) were prepared from the stock of 50 mM dissolved in slightly alkaline DDW. Throughout the process, overnight acid rinsed glass wares were used.

2.3. Synthesis, harvesting and purification of AgNPs

Silver nitrate (1mM) solution in DDW was prepared. The $AgNO_3$ and YPE were mixed at the ratio of 8:1 in an acid rinsed glass beaker of 500 ml capacity. The mixture was then subjected to rigorous stirring on a magnetic stirrer at room temperature. Various reaction parameters for silver nanoparticle synthesis such as molar concentrations (0.2–1 mM), pH of reaction medium (6–10), pure QDH concentrations (25–100 μM) and time of incubation (min) at constant temperature (25 ± 2 °C) were standardized using different sets of flasks to obtain the highest yield of AgNPs. Based on the experimental observations, pH 9 of the reaction mixture, 1 mM of $AgNO_3$, and 1:8 v/v ratio of YPE:$AgNO_3$ (1 mM) was found optimum for the bulk production of YPE-AgNPs (λ_{max} = 438 nm). Smilarly, pH 7, $AgNO_3$ (1 mM), and QDH (50 μM) was selected for the production of Q-AgNPs (λ_{max} = 431 nm). The pH of reaction mixture was adjusted using 1 N HCl and 1 N NaOH. The flask containing only $AgNO_3$ (0.2–1 mM) served as control for each reaction mixture under identical conditions. The synthesis was performed under dark conditions to avoid photo-activation of reducing solutions [31]. Aliquots of fabricated AgNPs were separated and stored under dark conditions for further stability studies [32]. Colored solution was centrifuged at 10,000 rpm for 30 min. Pellet was collected in a fresh tube and the supernatant was again centrifuged at the same speed and time. The pellet thus obtained was then subjected to washing with DDW followed by centrifugation at 10,000 rpm for 30 min. In the final step, pellet was dried in a vacuum oven (NSW, Delhi, India) at 50 °C for 24 h. The dark brown powder obtained was stored in acid cleaned glass vials for further characterization.

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