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Research Article

SILVER NANOPARTICLES: GREEN SYNTHESIS, OPTICAL PROPERTIES, ANTIMICROBIAL ACTIVITY AND ITS MECHANISM USING *CITRUS SINENSIS*

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ABSTRACT

Objective: Novelty and size specificity of silver nanoparticles (AgNPs) containing products gained popularity in today's world. The present investigation involves the biosynthesis of AgNPs from Ag nitrate using the peel extract of *Citrus sinesis*, is facile, worthwhile and promising approach toward environment protection.

Methods: The analytical techniques, such as ultraviolet-visible (UV-Vis) spectroscopy, Fourier transform infrared (FTIR), atomic force microscope (AFM), dynamic light scattering (DLS), scanning electron microscope (SEM) and energy-dispersive X-ray (EDX) analysis, were used to characterize synthesized nanoparticles. The antimicrobial activity of synthesized nanoparticle has also been examined on selected microbes.

Results: A UV-Vis spectrum shows peak absorption at 425 and 475 nm. FTIR spectroscopy confirmed the presence of protein as the stabilizing agent surrounding the AgNPs. The SEM and AFM results show average diameter of almost 80 nm, whereas DLS results show average diameter of the prepared nanoparticles 110 nm. The EDX spectrum confirmed the presence of an elemental Ag signal. Encouraging results were obtained against *Staphylococcus epidermidis* and *Bacillus cereus*.

Conclusion: AgNPs were found to be effective against selected microbes.

Keywords: Silver nanoparticles, Atomic force microscope, Energy-dispersive X-ray, Scanning electron microscope, *Staphylococcus epidermidis*, *Bacillus cereus.*

INTRODUCTION

Silver (Ag) is noble-metal nanoparticles which is known for its medical properties for over 2000 years and have substantial impact across a diverse range of fields, including catalysis [1], sensing [2], photochemistry [3], optoelectronics [4,5], energy conversion [6], and medicine [7]. The most of the chemical and physical methods of nano Ag production use toxic and hazardous chemicals which may pose potential threat to the environmental and are extremely expensive. The quest for environmentally friendly, non-toxic, and economically feasible technique led to the biomimetic production of Ag nanoparticles (AgNPs) which is synthesis AgNPs using biological methods. The "greener synthesis" of nanoparticles proven to be better method due to slower kinetics, better manipulation, control over crystal growth, and their stabilization. Thus, biological method has motivated an upsurge in research to synthesis nanoparticles of controlled shape and size for various nanotechnological applications. Ag-based compounds have been used in many antimicrobial applications as they are highly toxic to microorganisms [8,9]. This property of Ag plays multiple roles and emerged as boon in the medical field.

Staphylococcus epidermidis, a Gram-positive bacterium, belonging to the genus *Staphylococcus* [10]. It is part of the normal human flora, typically the skin flora. Infections due to *S. epidermidis* have also been steadily raising and forms biofilms on plastic devices placed within the body [11]. This occurs most commonly on intravenous catheters and on medical prostheses [12]. It also causes endocarditis, most often in patients with defective heart valves. Another Gram-positive, rod-shaped, endospore forming, facultative erobic bacterium is *Bacillus cereus*, is an opportunistic food borne pathogen, which causes two distinct types of food poisoning, i.e. diarrhea and emesis. It is also responsible for spoilage of different food products [13]. *Fusarium acuminatum* is toxigenic species that contaminate cereal crops from diverse climatic regions. Therefore, the present study deals with synthesis approach which is simple and "green" for the synthesis of metallic nanostructures of noble metal, i.e., Ag using peel of *Citrus sinesis* (commonly known as orange). The synthesized AgNPs were optimized, characterized, and its effect on microbes was then studied for *S. epidermidis B. cereus* and fungi *F. acuminatum.*

Green technique based synthesis of AgNPs using peel extract of *Citrus sinensis* is simple and non-toxic technique which utilizes peel of *C. sinensis* (orange) which thrown as waste in dustbins to generate economically feasible and environment friendly AgNPs.

METHODS

Selection of plant material

Present study was carried out using peel powder of *C. sinensis.*

Phytochemical screening

Phenol test

Formation of intense color in the alcoholic extract of plant material in addition of 1-2 drops of 1% $FeCl_3$ solution.

Saponin test

Boil freshly chopped material in a test tube with water for 1 minute. Cool the tube and set aside for 5 minutes. Stable foam of about 2 cm or more will be formed.

Steroid test

2 ml of acetic anhydride was added to extract containing 2 ml of H_2SO_4. The color of solution changed from violet to blue or green in sample.

Flavonoid test

A small quantity of the extracts is heated with 10 ml of ethyl acetate in boiling water for 3 minutes. The mixture is filtered differently and

the filtrate was shaken with 1 ml of dilute ammonia solution (1%). The layers were allowed to separate. A yellow coloration was observed at ammonia layer. This indicates the presence of the flavonoid.

Tannins test

Extract is boiled with 5 ml of 45% ethanolic solution for 5 minutes. Cool the tubes and filter the solution. 1 ml of filtrate is diluted using distil water, and 2 drops of $FeCl_3$ was added. A transient greenish to black indicates tannin.

Alkaloids test

Methanolic extract was warmed with 2% H_2SO_4 for 2 minutes. It is filtered, and few drops of Dragendroff's reagent were added and indicated the presence of alkaloids.

Preparation of orange peel extract (OPE)

Fresh orange peel was collected and washed thoroughly with water to remove impurities and then dried in oven. Dried peels were chopped into powdered form. The 5g of powdered form was suspended in 100 ml boiling distilled water for 15 minutes. This mixture was then filtered and filterate was centrifuged (eppendrof 5810 R) at 9000 rpm for 15 minutes to get the extract. Supernatant was taken out and its volume was adjusted to 100 ml by adding distil water. Furthermore, this extract was filtered through Whatman filter paper No. 1. The extract so obtained was OPE, should be used within a week for AgNPs synthesis.

Synthesis of AgNPs

For the synthesis of AgNPs different concentration of Ag nitrate ($AgNO_3$) (1, 2, and 5 mM) from Fischer Scientific, has been used. The OPE (5 ml and 10 ml) was added to 50 and 100 ml of 1, 2, and 5 mM concentration solution of $AgNO_3$. The reaction was carried out at room temperature. The change in color indicates synthesis of AgNPs. The solution was centrifuged at 9000 rpm for 15 minutes to separate AgNPs. AgNPs was obtained in the form of pellet. The pellet was resuspended in distil water to remove any uncoordinated biological molecule. This process was repeated two more times. The purified pellet was then transferred into small petriplate and oven dried at 60°C for 24 hrs. The dried AgNPs were scrapped for characterization.

Antimicrobial activity of AgNPs synthesized from *C. sinesis* peel aqueous extract

Test microorganisms

Two bacterial strains *S. epidermidis* (Microbial Type Culture Collection [MTCC] Code 2639), *B. cereus* (MTCC Code 9017), and fungi *F. acuminatum* (MTCC Code 9989) were used in the present study; all strains were obtained from IMTECH Chandigarh, India. These test cultures were grown in nutrient agar medium, nutrient broth (Hi media, M002), at 37°C and potato agar slants at 4°C.

The disc diffusion method [14] was followed for testing AgNPs at a concentration 50 µg/ml and 100 µg/ml. The discs were soaked with different solution having different concentration of AgNPs. Then, the discs were air dried in sterile condition. The plates containing nutrient agar media were prepared by swabbing them with the microbial cultures. Prepared discs were placed on plate. The plates were incubated at 37°C for 24-48 hrs. Then, the maximum zone of inhibition were observed and measured for analysis against each type of test microorganism.

Characterization of AgNPs

Optical absorbance of the synthesized AgNPs was performed using a ultraviolet-visible (UV-Vis) spectrophotometer (UV-vis scan 167 from Systronic) between the wavelengths of 300 and 800 nm as a function of time at a resolution of 1 nm.

Dried and powdered AgNPs were palleted with potassium bromide (KBr) (1:10 proportion) to determine the binding properties of AgNPs synthesized by OPE using Fourier transform infrared spectroscopy (FTIR) analysis (SHIMADZU-8400S). The spectra were recorded in the wavenumber range of 400-4000/cm. Further biosynthesized AgNPs were characterized using electron microscope that images a sample by scanning it with a high-energy beam of electrons in a raster scan patterns. In present study, ZEISS supplied scanning electron microscope (SEM) was used. By measuring energies of the X-rays and auger electrons emitted by biosynthesized AgNPs energy-dispersive X-ray spectroscopy (EDX) spectrum was recorded from ZEISS SEM instrument. A thin film of AgNPs was prepared by dropping 100 µl of sonicated sample in alcohol on the slide and was allowed to dry for 5minutes. The slide was then scanned with the atomic force microscope (AFM) (Nanosurf).

Further particles size determination of biosynthesized AgNPs was done using dynamic light scattering (DLS) (Zetasizer, Malvern). Shining a monochromatic light beam, such as a laser, onto a solution with spherical particles in Brownian motion causes a Doppler shift when the light hits the moving particle, changing the wavelength of the incoming light. This change is related to the size of the particle. It is possible to compute the sphere size distribution and gives a description of the particle's motion in the medium, measuring the diffusion coefficient of the particle and using the autocorrelation function.

RESULTS

The current study was undertaken to exploit the hitherto un-utilized plant sources in the development of AgNPs. The plant powder was subjected to phytochemical screening using the conventional methods to test for the alkaloids, tannins, flavonoids, steroids, and phenol were found to be present (Table 1).

UV-Vis analysis

After addition of peel extract to $AgNO_3$ solution initial lemon yellow changes to final reddish brown suggest AgNPs (Fig. 1) [15-17]. Similar results were obtained in previous studies [18-22] and hence confirmed the completion of reaction between peel extract and $AgNO_3$. Sample analysis for synthesis of AgNPs was carried out at 30, 40, 50, and 60 minutes are shown in Fig. 2. Absorption spectrum of AgNPs formed in the reaction media has absorption maxima at 425 and 475 nm.

Table 1: Phytochemical investigation of active constituents in plant powder

S. No	Phytochemicals	*Citrus sinensis*
1	Alkaloids	+
2	Tannins	+
3	Flavonoids	+
4	Steroids	+
5	Phenol	+
6	Saponin	–

+: Positive, –: Negative, *C. sinensis: Citrus sinensis*

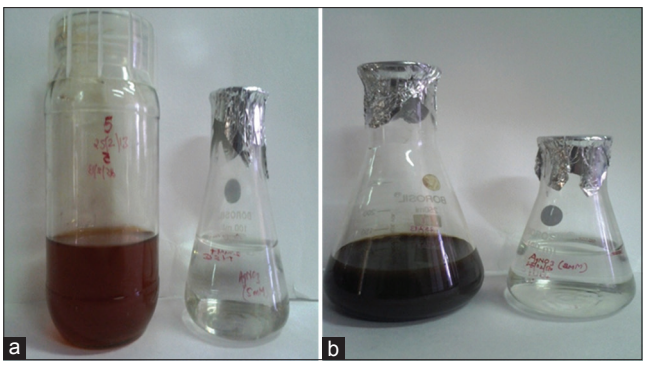

Fig. 1: Change in color after addition of orange peel extract to 5 mM and 2 mM $AgNO_3$ solution. (a) Biosynthesized silver nanoparticles (left) and (b) silver nitrate solution (right)

FTIR analysis

FTIR study of peel powder showed sharp absorption peaks located at about 1500 and 1650/cm represents C-C stretch (in-ring) and N-H bend may be assigned to the amide I bond of the proteins and peaks at 3430/cm are assigned to OH stretching in alcohol and phenolic compounds [23] as shown in Fig. 3a.

The FTIR analysis carried out to characterize the AgNPs obtained from of plant extract is shown in Fig. 2. AgNP solutions, prominent bands of absorbance were observed at around 650, 1500, 1600, 1550-1650, 3500/cm. The observed peaks denote -C-O-C-, ether linkages, -C-O-, germinal methyl or strong stretching due -C=C- bond of aromatic rings and alkyne bonds, respectively. The strong peak absorption at 3200-3500/cm may result from the alcohol/phenol O-H stretch as shown in Fig. 3b.

SEM analysis

The bright illuminated portion against dark background was obtained from SEM analysis of the biosynthesized AgNPs are shown in Fig. 4. It is seen that AgNPs of square and rectangle shapes were obtained in case of peel extracts acting as reducing and capping agents. Formation of such shapes may be due to availability of different quantity and nature of capping agents present in the peel extract. This is also supported by the shifts and difference in areas of the peaks obtained in the FTIR analysis. The particles were in range from 40 to 155 nm. The average size of the particle was found to be 80 nm.

DLS

DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering, is a technique used to determine the size distribution profile of nanoparticles in suspension. The mean average size of AgNPs comes out was 110 nm as shown in Fig. 5. The shape and structure could be controlled by changing the reaction temperature and peel extract concentration.

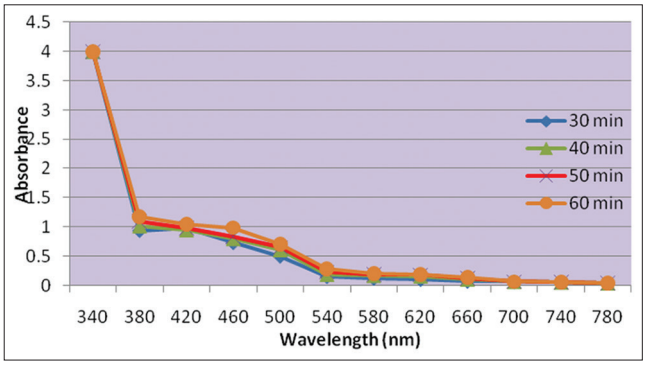

Fig. 2: Ultraviolet-visible spectra of biosynthesized silver (Ag) nanoparticles at 5 mM concentration of Ag nitrate

EDX analysis

Spectrum present three peaks located between 0 and 4 KeV as shown in Fig. 6. Maxima are directly related to the Ag characteristic lines K and L. Almost at 0 KeV, there is peak of chlorine followed by carbon and then oxygen. Carbon and oxygen in the samples confirm the presence of stabilizers composed of alkyl chains. Quantitative analysis of EDX analysis proved high Ag content (72.18%) in examined samples.

AFM

The AgNPs were characterized three-dimensionally by AFM for its detail topological evaluation (size, morphology, and agglomeration). Area of sample scanned was 50 µm × 49.5 µm with silicon cantilevers at tip height 10-15 nm, contact mode with constant force of 0.02-0.77 N/m. AFM detects agglomerated and distinct nanostructures (nanoparticles). The particles were irregular and some are spherical in shape and mostly dispersed; although in some places, nanoparticles were found to be in aggregates. The graph depicting the profile of the particles under AFM shows that particles were in range from 50 to 155 nm in height. Surface analysis reveals irregular AgNPs (Fig. 7). AgNPs so formed and its agglomeration were clearly observed in Fig. 7. The average size of the AgNPs ranges in size from 76 nm.

Control of reaction rate and particle size

Further by changing the composition of the reaction mixture the possibility of controlling the reaction rate and particle size was analyzed. Fig. 8 shows the time courses of AgNPs formation with different amount of OPE keeping concentration of $AgNO_3$ constant and vice-versa. The reaction rate was highest at 20% peel broth concentration. An observation shows that the average particle size increases with increasing the leaf broth concentration. Sub-micron scale particles between 100 and 500 nm were obtained with high concentrations of peel extract.

Mechanism of AgNPs formation

AgNPs synthesized using orange peels extract in which tannins and flavonoids are main component responsible for bio-reduction. They are water soluble polyphenolic compounds. They cause proteins coagulation and chelation of metal ions. They are also powerful antioxidants (reductants) and results in transfer of electrons to hydroxyl groups by eliminating reactive unpaired electrons in oxygen radicals. They cause reduction of Ag^+ ions at the atomic Ag. Oxidation of phenol results in quinones or quinoid structures in the case of tannins.

The higher total phenolic content in peel extract facilitates the reduction of Ag ions to nanoscale-sized Ag particles due to the electron donating ability of these phenolic compounds. Moreover, the quinoid compound

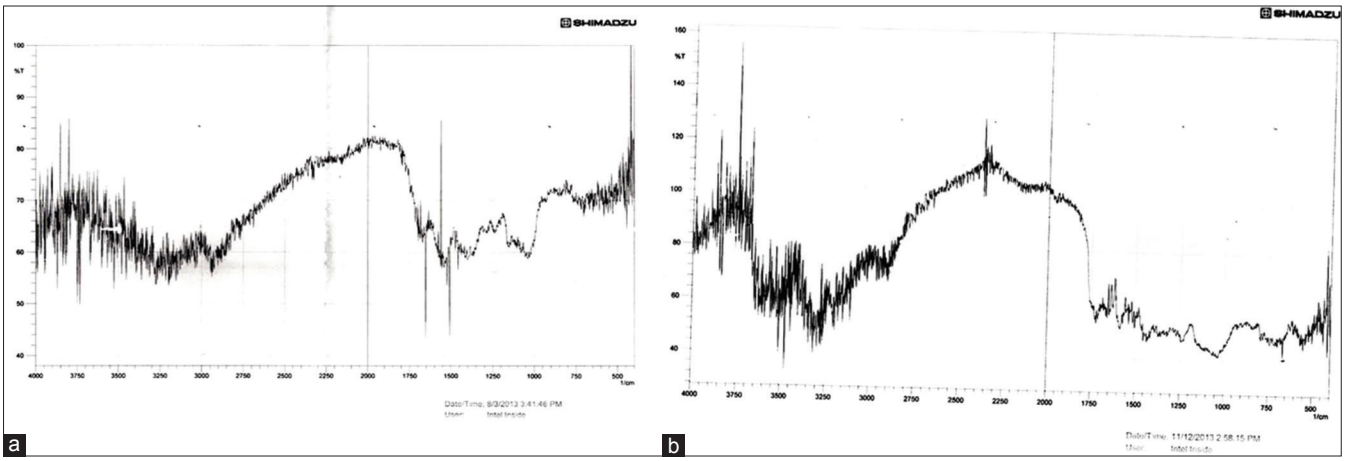

Fig. 3: Fourier transform infrared analysis, (a) Peel powder and (b) biosynthesized silver nanoparticles

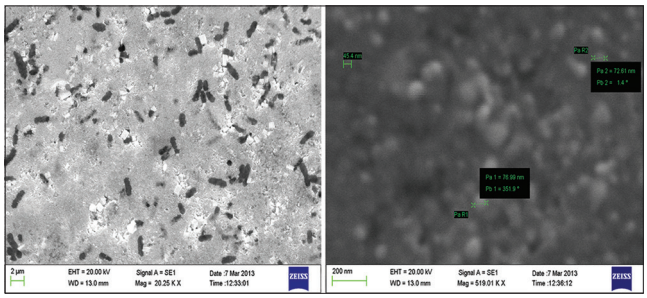

Fig. 4: Scanning electron microscope image of silver nanoparticles showing different shape and size nanoparticles

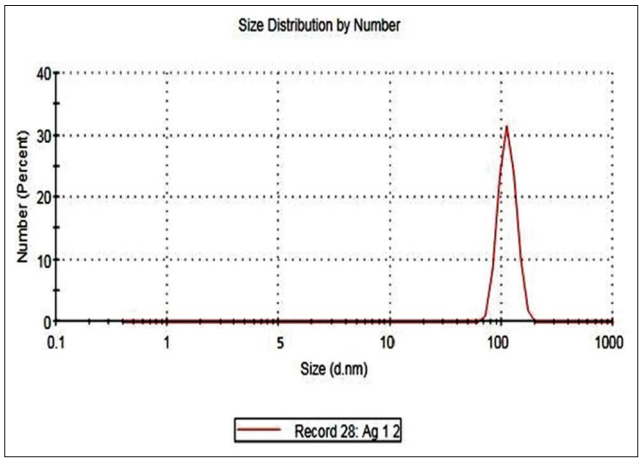

Fig. 5: Dynamic light scattering graph of silver nanoparticles showing size distribution intensity

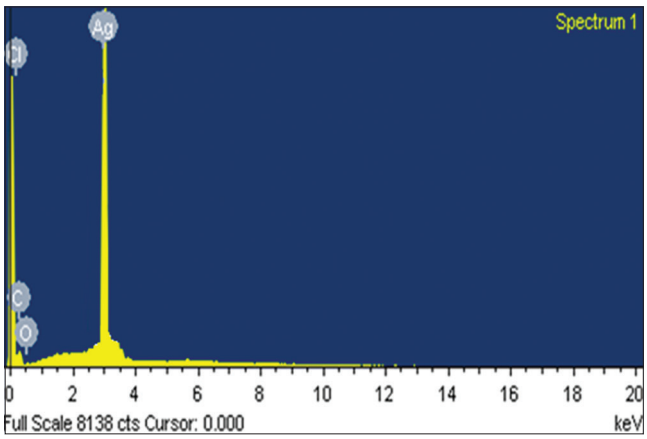

Fig. 6: Energy-dispersive X-ray spectrum showing peak of carbon, oxygen, chlorine, and elemental silver

produced due to the oxidation of the phenol group in phenolics can be adsorbed on the surface of nanoparticles, accounting for their suspension stabilization [24].

Antimicrobial activity of AgNPs against human and plant pathogenic bacteria and multidrug-resistant bacteria

The human bacteria *S. epidermidis*, *B. cereus* and fungi *F. acuminatum* were used to assay the antimicrobial activity of the AgNPs. *S. epidermidis* plates showed inhibition zones which increased with the increase in concentration of AgNPs (100 µg/ml) as shown in Fig 9. Similar results were obtained in case *B. cereus.* However, AgNPs are not to be effective against *F. acuminatum*.

Mechanism of action

Anchoring ability of AgNPs to the cell wall and later on its permeation into the bacterial cell results in altered cell membrane and leading to cell death. Indentation in a surface of cell [25], and free radicals could be alternate pathway for cell death [26,27]. Interaction of Ag ions released by nanoparticles with thiol group of many enzymes leading to its inactivation could be another proposed mechanism of action of AgNPs [28,29]. Nanoparticles of Ag act on soft bases within the cell like sulfur and phosphorus and lead to cell death by destroying DNA [30,31].

Bacterial cell interaction with nanoparticles can transmogrify the signal transduction pathway. Alteration in phosphotyrosine residues of peptide due nanoparticles inhibit bacterial growth. Further investigation is essential/indispensable in this area to understand it thoroughly and establish the claims.

DISCUSSION

AgNPs provide good platform and promising applications as an antibacterial agent. Surface plasmon resonance is responsible for absorption peak after the addition of plant extract to $AgNO_3$ solution at room temperature. However, large and dispersed (poly) AgNPs so obtained may be due to slower rate of reaction between plant extract and AgNO3, which was further clear from UV-Vis graph showing broadening of the peak [21,22]. UV-Vis absorption peak so obtained is nearby to previous reported work [32]. The solution AgNPs was found to be stable even after 24 hrs. The FTIR characteristic absorption peak shows stretching vibrational bands of flavonoids and terpenoids [33,34] denoting that they play crucial role in capping and stabilizing nanoparticles. These are derived from water soluble compounds such as flavonoids, alkaloids, and polyphenols present in peel. Biological components are known to interact with metal salts via these functional groups and mediate their reduction to nanoparticles [35]. Further spectroscopic studies also shows that protein could also be responsible for stabilizing the synthesized AgNPs as peptides of protein and carbonyl groups of amino can bind to metals strongly [36] by forming covering around the metal nanoparticles and thus leads to the stability [37].

The EDX spectra of biosynthesized AgNPs shown in Fig. 6. The EDX profile confirms the synthesis of Ag with strong Ag signal with

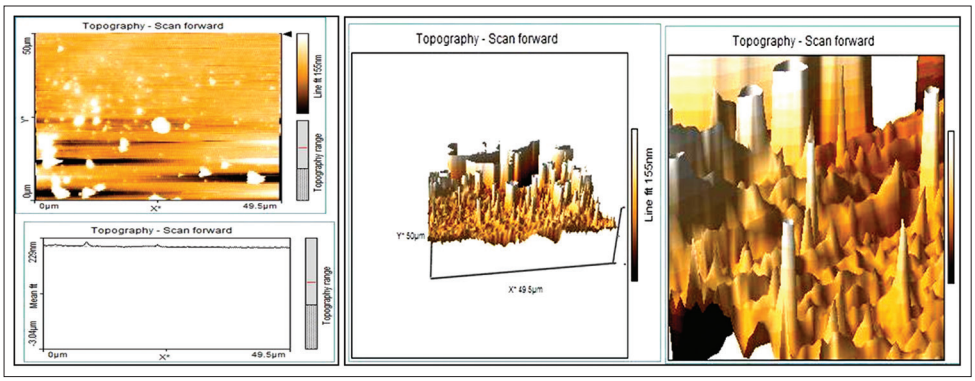

Fig. 7: Atomic force microscope images of silver nanoparticles from orange peel extract

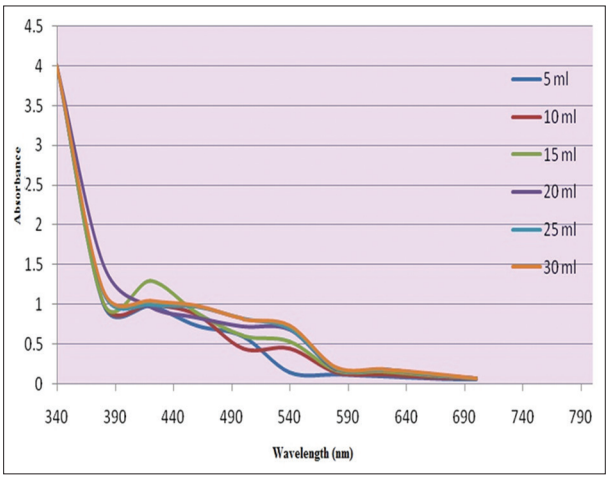

Fig. 8: Ultraviolet-visible absorption of silver nanoparticles at different concentrations of orange peel extract

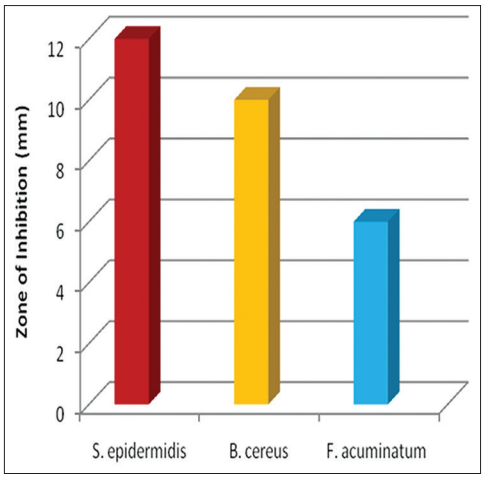

Fig. 9: Zone of inhibition formed at 100 µg/ml concentration of silver nanoparticles against selected microbes

low oxygen and carbon peaks, which could be attributed from the biomolecules bound to the surface of the AgNPs. Appearance of carbon and oxygen peaks may be due to the distil water utilized for biosynthesis of AgNPs. Studies have shown that plant based nanoparticles are encapsulated by layer of capping agent from extract and found to be remain unchanged in solution for up to longer period of time after its biosynthesis [38,39]. This is another merit associated with use of the biological method over chemical method. The hydrodynamic radius could be the reason for larger particle size in case of DLS as compared to the particles size measured from AFM micrograph [40].

Different size of AgNPs suggested that too many reducing agents cause aggregation of the Ag particles synthesized possibly due to the interactions between anchored capping agent to the particles surface and secondary reduction process on the surface of the preformed nuclei.

CONCLUSION

The synthesis of AgNPs using the peel extract of *C. sinensis* is the cost-effective reduction method and has shown a potential to be used in place of chemical-based antibiotics. There are fewer chances of pathogenic bacteria to develop resistance against biosynthesized AgNPs and may play crucial role in fields such as medical devices and antimicrobial systems.

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