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GREEN SYNTHESIS OF SILVER NANOPARTICLES BY USING STEM DERIVED CALLUS EXTRACT OF BITTER APPLE (*CITRULLUS COLOCYNTHIS)*

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Plant mediated synthesis of metallic nanoparticles is an increasing commercial demand due to the wide applicability in various areas such as electronics, catalysis, chemistry, energy, cosmetics and medicine. In the present investigation, synthesis of silver nanoparticles by using stem derived callus extracts of *Citrullus colocynthis* (L.) Schrader. The extract incubated with $AgNO_3$ showed gradual change in the colour of the extract from greenish to reddish brown it indicate the silver nanopaticles synthesis. The shape of the SNP synthesized by stem derived callus extract was spherical and was found to be in the range of 75 nm by AFM. FTIR absorption spectra conclude that the compounds attached with silver nanoparticles could be polyphenols with aromatic ring and bound amide region. The novel silver nanoparticles exhibited a tremendous antibacterial activity; it showed the maximum activity against bioflim bacteria such as *E.coli* (10.1 mm), *V. paraheamolyticus* (10.1 mm), *P. aeruginosa* (8 mm), *Proteus vulgaris* (9 mm) and *L. monocytogens* (8 mm) and also observed that it showed no activity against *Proteus mirabilis*, *Salmonella enteritidis*, and *Staphylococcus aureus.* To the best of our knowledge this is the first report on the antibacterial activity of silver nanoparticles against bioflim forming bacteria.

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1. Introduction

Citrullus colocynthis belong to the family of cucurbitaceae are purgative and used for the treating mamilities, jaundice and urinary disease [1]. *Citrullus colocynthis* is mainly cultivated for its edible fruits and seeds which are rich in oil and proteins [2-3]. The therapeutic potentials *viz*., antimicrobial [4], anti inflammatory [5], anti diabetic [6] and anti oxidant [7] effect of *Citrullus colocynthis* have reported in our laboratory. Nanoparticles usually referred as particles with a size up to 100nm [8]. Nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. Specific surface is relevant for catalytic reactivity and other related properties such as antimicrobial activity in silver nanoparticles. As specific surface area of nanoparticles is increased, their biological effectiveness can increase in surface energy [9]. Silver has long been recognized as having an inhibitory effect towards many bacterial strains and micro organisms commonly present in medical and industrial processes [10]. The most widely used and known applications of silver and silver nanoparticles are in medical industry. These include topical ointments and creams containing silver to prevent infection of burns and open wounds [11]. Production of nanoparticles can be achieved through different methods. Chemical approaches are the most popular methods for the production of nanoparticles. However, some chemical methods cannot avoid the use of toxic chemicals in the synthesis protocol. Since noble metal nanoparticles such as gold, silver and platinum nanoparticles are

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widely applied to human contacting areas, there is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals. Biological methods of nanoparticles synthesis using micro organisms [12], enzyme [13], and plant or plant extract have been suggested as possible ecofriendly alternatives to chemical and physical methods. Using plant for nanoparticles can be advantageous over other biological processes by eliminating the elaborate process of maintaining cell culture [14]. It can also suitably scaled up for large- scale synthesis of nanoparticles. The synthesis of pure metallic nanoparticles of silver by the reduction of Ag^{+} and Au^{3+} ions using Neem (*Azadirachta indica)* leaf broth [15]. However, little has been carried out about engineering approaches such as rapid nanoparticles synthesis using plant leaf extracts and size control of the synthesized nanoparticles. The times required for more than 90% reduction of Ag^{+} and Au^{3+}ions using Neem leaf broth were about 4 and 2 h, respectively. If biological synthesis of nanoparticles can compete with chemical methods, there is a need to achieve faster synthesis rates. The exact mechanism of silver nanoparticles synthesis by plant extracts is not yet fully understood. Only participation of phenolics, proteins and reducing agents in their synthesis has been speculated. In the present study, we screened coastal sand dune species *Citrullus colocynthis* leaf extracts for extracellular nanoparticles synthesis, characterized by using UV- visible spectroscopy, SEM, AFM and FT-IR.

2. Experimental

2.1 Plant material and preparation of the extract

Fresh *Citrullus colocynthis* leaves were collected from the Southeast coast of Parangipettai (Tamil Nadu) India. The specimen was certified by Botanical Survey of India (BSI) Coimbatore, and documented in the Herbaria of C.A.S. in Marine Biology, Annamalai University, India, during 2010. The experimental chemicals were purchased from Sigma Chemicals (Mumbai).

2.2 Sample preparation for synthesis of Silver Nanoparticles

One month old compact, hard greenish white callus derived from stem explants was used to obtain the callus extract in our lab [16]. The callus was washed twice with sterile distilled water to remove medium components before grinding. Approximate 20 g of callus was crushed in 100 ml of sterile distilled water in mortar and pestle. The resulting extract was filtered through filter paper (What man No.1) and used for the synthesis of silver nanoparticles. 10 ml suspension of callus culture was added to 90 ml aqueous solution of silver nitrate (1mM) solution separately for reduction in to Ag^{+} ions and incubated at room temperature (35°C) for about 24 hours. The primary detection of synthesized silver nanoparticles was carried out in the reaction mixture by observing the colour change of the medium from greenish to dark brown. After 5h of incubation the silver nanoparticles were isolated and concentrated by repeated (4-5 times) centrifugation of the reaction mixture at 10,000×g for 10 min. The supernatant was replaced by distilled each time and suspension stored as lyophilized powder for the optical measurements [17]

2.3 Atomic Force Microscope

Purified SNP in suspension was also characterized their morphology using a VEeco diNanoscope 3D AFM (Atomic Force Microscope). A small volume of sample was spread on a well-cleaned glass cover slip surface mounted on the AFM stub, and was dried with nitrogen flow at room temperature. Images were obtained in tapping mode using a silicon probe cantilever of 125μm length, resonance frequency 209-286 kHz, spring constant 20-80 nm^{-1} minimum of five images for each sample were obtained with AFM and analyzed to ensure reproducible results.

2.4 Fourier Transform Infra Red Spectroscope

To identify Silver nanoparticles associated biomolecules, the Fourier transform infra red spectra of washed and purified Silver nanoparticles powder were recorded on the Nicolet Avatar 660 FT-IR Spectroscopy (Nicolet, USA) using KBr pellets. To obtain good signal to noise ratio, 256 scans of Silver nanoparticles were taken in the range of 400-4000 cm^{-1} and the resolution was kept as 4 cm^{-1}

2.5 Isolation of Biofilm Bacteria from Boat Hull

The present study samples were collected from the southeast coast of Tamil Nadu (Vellar estuary, Parangipettai, Lat 11º26'N; Log 79º46' E) during the period of April-June 2010. The bottom of the boat was gently swabbed with a sterile cotton swab, placed in tubes containing 10 mL sterile water. Then they were inoculated in specific media for the isolation of microbes. The bioflilm bacterial strains used in the antibacterial assay were isolated by the pour plate technique [18].

2.6 Antibacterial Assay

Antibacterial activity of the silver nanoparticles was assessed using the standard agar diffusion method with 6mm diameter Whatmann No.1 filter paper discs (Becerro *et al.,* 1994). In this method 50 μl of silver nanoparticles prepared from callus extract was mixed in 1 ml of distilled water and applied to sterile paper discs of 6mm diameter and standard antibiotic disc (ampicillin and tetracycline) used for control. Zobell marine agar was used for the antimicrobial test. Before the antibacterial assay the biofilm forming bacteria (*P. aeruginosa, P. vulgaris, P. mirabilis, E. coli, S. enteritidis, S. aureus, L. monocytogens and V. paraheamolyticus*) were inoculated into the Zobell marine agar plates and incubated at 27ºC for 24 hours. Inhibition of zone was measured after 24-48 h of inhibition.

3. Results

The callus extract was used for the synthesis of silver nanoparticles. The reaction started with in first hour of the incubation with silver nitrate (1 mM). This was confirmed by the appearance of brown colour in the reaction mixture**.** The shape of the SNP synthesized by stem derived callus extract was spherical and was found to be in the range 75 nm by AFM (Fig. 1). Finally, confirmed the synthesis of spherical silver nanoparticles in the reaction mixture. The larger size of the nanoparticles might be due to the capping of nanoparticles by polyphenols with aromatic ring and bound amide as confirmed from FT-IR analysis.

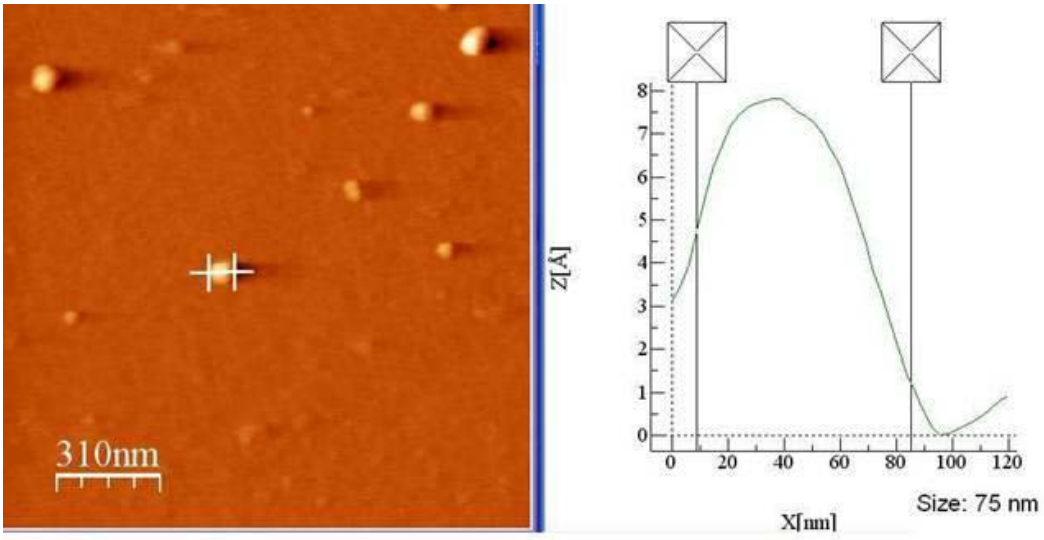

Fig. 1. AFM images of silver nanoparticles

Representative absorption spectrum of the nanoparticles obtained in the present study is presented in (Figure 2). Among them, the absorption peak at 1020 cm^{-1} can be assigned as absorption peaks of C-O-C- or –C-O-, also the peak at 1020-1091 cm^{-1} corresponds to C-N stretching vibrations of aliphatic amines or to alcohols or phenols representing the presence of polyphenols [19]. The absorbance peak at 1265 and 1384 – 1460 cm^{-1} correspond to the amide III and II group respectively. The peak at 1624 cm^{-1} is associated with stretch vibration of –C=C- [20] and is assigned to the amide 1 bonds of proteins. The absorption at about 1384 cm^{-1} is notably enhanced indicating residual amount of NO_3 in the solution [21]. The peak at 1539 cm^{-1} may be assigned to symmetric stretching vibrations of –COO- (carboxyl ate ion) groups of amino acid residues with free carboxyl ate groups in the protein [22]. The peak at 3427 cm^{-1} indicates polyphenolic OH group along with the peak of 882 cm^{-1} which represents the aromatic ring C-H vibrations, indicate the involvement of free catechin [23]. This suggests the attachment of some polyphenolic components on to silver nanoparticles. This means the polyphenols attached to silver nano particles may have atleast one aromatic ring. The peaks at 1000-1200 cm^{-1} indicate C-O single bond and peaks at 1620-1636 cm^{-1} represent carbonyl groups (C=O) from polyphenols such as catechin gallate, epicatechin gallate and theaflavin [24]. Result suggests that molecules attached with silver nanoparticles have free and bound amide group. These amide groups may also be in the aromatic rings. This concludes that the compounds attached with silver nanoparticles could be polyphenols with aromatic ring and bound amide region.

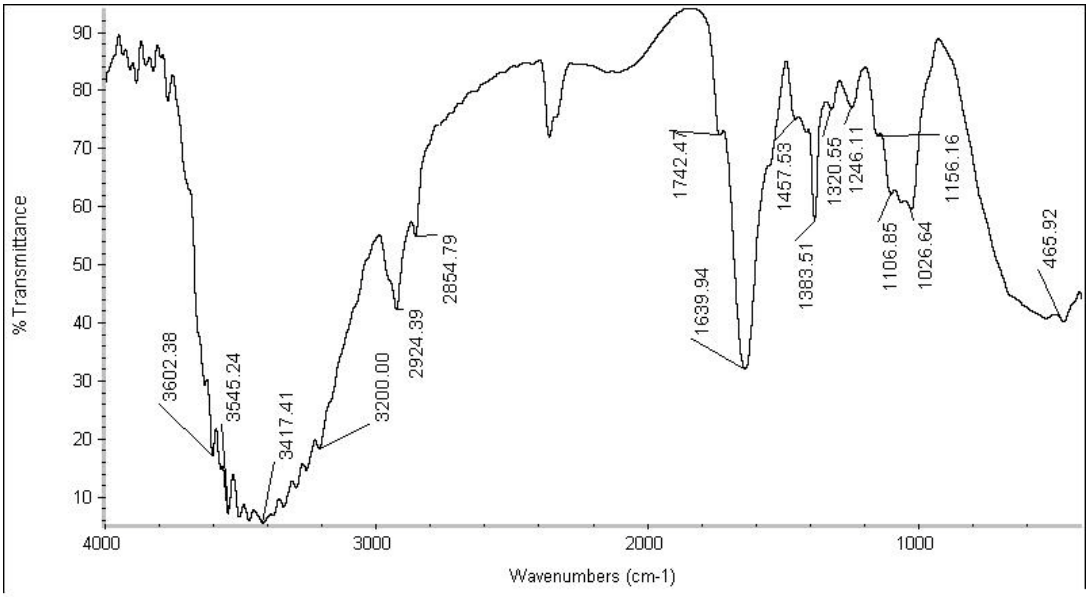

Fig. 2. FT-IR images of silver nanoparticles

3.1 Identification of Biofilm Microorganisms

The incidence of total bacterial population was increased during every month intervals on surface of boat hull. The count varied between 12×10^6 to 45×10^6 CFU $mL^{1.}$ The biofilm forming bacteria (*Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis, E.coli, Listeria monocytogens, Salmonella enteritidis, Staphylococcus aureus and Vibrio paraheamolyticus*) were isolated from the boat hull and identified using Bergey's Manual.

3.2 Antibacterial Assay

The callus derived silver nano particles was showed the maximum activity against *E.coli* (10.1 mm), *V. paraheamolyticus* (10.1 mm), *P. aeruginosa* (8 mm), *Proteus vulgaris* (9 mm) and *L. monocytogens* (8 mm) and also observed that it showed no activity against *Proteus mirabilis, Salmonella enteritidis,* and *Staphylococcus aureus.* The antimicrobial properties of silver compounds and silver ions had been historically recognized and applied in a wide range of applications from disinfecting medical devices and home appliances to water treatment [25]. Silver ion and silver based compounds are highly toxic to micro organisms, showing strong biocidal effect against microbial species. The silver nano particles produced by microbes and plant extracts are known to exhibit potent antimicrobial activity. A similar observation has been made with the silver nano particles produced by callus extract to have antimicrobial activity against the biofilm forming bacteria (*E. coli, V. paraheamolyticus, P. aeruginosa, Proteus vulgaris* and *L. monocytogens).*

4. Conclusions

Our investigation revelas, the bioreduction of aqueous Ag^+ ions by the callus extract of the *Citrullus colocynthis* has been demonstrated. The reduction of the metal ions through the callus extracts leading to the formation of silver nanoparticles of fairly well – defined dimensions. This green chemistry approach toward the synthesis of silver nanoparticles has many advantages. applications of such eco- friendly nanoparticles in bactericidal, wound healing and other medical and electronic applications make this method potentially exciting for the large- scale synthesis of other inorganic materials (nanoparticles).

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