



Research Article

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In Vitro Studies of Skin Burn Infections Using *Casuarina Equisetifolia* L. Fabricated Silver Nanoparticles

Sanjay Moharekar¹, Aparnnaa Kulkarni², Mahadev Uplane³, Shubhangi Dushing¹, Bhakti Dayma¹ and Shubhangi Moharekar^{*1,4}

¹Department of Biotechnology, New Arts, Commerce and Science College, Ahmednagar, India.

²Department of Statistics, New Arts, Commerce and Science College, Ahmednagar, India.

³Department of Instrumentation Science, Savitribai Phule Pune University, Pune, India.

⁴Savitribai Phule Pune University, Pune, India.

***Corresponding author:** Shubhangi Moharekar, Savitribai Phule Pune University, Pune and Department of Biotechnology, New Arts, Commerce and Science College, Ahmednagar, India.

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Abstract

In present work, Silver Nanoparticles (AgNPS) were synthesized by biological as well as chemical method. Biosynthesis of silver nanoparticles was done using dried and burned fruit powder of *Casuarina equisetifolia*. The characteristics of synthesized nanoparticles were confirmed by UV-Visible spectroscopy, X-Ray Diffraction (XRD) and Scanning Electron Microscopy (SEM). The size of biologically synthesized nanoparticle was comparatively smaller than chemically synthesized one. The biologically synthesized AgNPs showed remarkable antibacterial activity against five pathogenic bacteria isolated from burn patient's skin viz. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter* sp. and *Klebsiella pneumoniae*. Based on these findings, it was concluded that the present study provides potential of biologically synthesized AgNPs as an effective antibacterial agent against skin pathogens and hence can be used for medical application to prevent skin burn infections. These results clearly indicate that AgNPS could provide alternative topical therapy in treatment of skin burn infection.

Keywords: Antibacterial agent, *Casuarina equisetifolia*, Silver nanoparticle, Skin Burn Infections

Introduction

Nanotechnology is the study of nano sized materials dealing with their synthesis and wide range applications. Various properties of a material depend on its size and shape, as size of material reduces to nano-scale its properties do change. Noble metal nanoparticles such as gold, silver, platinum and palladium have been most effectively studied [1-3]. These nanoparticles can be synthesized

using physical, chemical and biological methods. Chemically synthesized nanoparticles are not favourable for biomedical application due to its toxicity and flammable nature of starting chemical compounds. The biological approach to synthesize metal nanoparticles is of great scientific interest and current need of nanotechnology research. Use of biological materials such as microorganisms,



plant extract or biomass for nanoparticle synthesis is an eco-friendly and economic alternative to chemical and physical methods [4-6]. The presence of diverse phytochemical constituents present in plant material may play role in biosynthesis of metal nanoparticles. In recent years, several plants have been reported for synthesis of silver, copper and gold nanoparticles such as extracts of neem, aloe vera, wheat, lemongrass, tamarind, ginger and garlic [7-12].

The emerging infections in burn patient represent a serious therapeutic challenge because bacteria leukocidin and tissue-destrorying enzymes which leads to extensive skin loss and heavy contamination (Kang 2016). As a result, wound healing process get altered or delayed. Therefore, topical antimicrobial therapy to control microbial colonization and proliferation of microbial pathogens including multidrug-resistant organisms is most important to take care of burn wound. It has been estimated that in developing countries about 75% of the mortality associated with burn injuries is related to sepsis [13]. The reason behind this phenomenon is the increasing resistance to antibiotics in infectious bacteria due to its overuse; therefore, there is a need for an alternative therapy. Nanoparticles can serve as potent alternative to the antibiotics, because nanoparticles have antibacterial and antifungal activity [14]. *Casuarina equisetifolia* is traditionally used as a medicinal plant [15,16], uses like anti-inflammatory, antibacterial, anti-diabetic, hepatoprotective activity, antioxidant, antispasmodic etc [17]. The leaf of *C. equisetifolia* are used as folk medicine for treating nervous disorder, diarrhea, stomach-ache, cough, ulcers, diabetes etc [8-10]. The fruit of *C. equisetifolia* is woody and oval structure made up of numerous carpels, each with single seed and small wing. The fruits of *C. equisetifolia* plant were used in the given study because tribal people used it to treat skin burns because it is well known that plants-derived NPs are less harmful side effects than that of chemically synthesized NP [18]. To my knowledge for the first time, the present work reports green synthesis of AgNPs from medicinally valuable plant *C. equisetifolia* fruit extract as a reducing agent. In the present work, we investigated the biosynthesis of AgNPs using fruit of *Casuarina equisetifolia*. The properties of obtained AgNPs were characterized by UltraViolet-Visible (UV-Vis) spectroscopy, Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD) techniques. This work provided a potent application of AgNPs to serve as an alternative to antibiotics against skin burn pathogens.

Materials And Methods

Materials

Fruits of *Casuarina equisetifolia* were collected from campus of NACAS College, Ahmednagar. Dried fruit powder and burned fruit powder of above plant were prepared and further used for stock preparation. Analytical grade silver nitrate (AgNO_3) was purchased from Merck chemicals. Autoclaved distilled water was used for preparation of 100mM AgNO_3 stock solution.

Preparation of Plant Extract

Collected dried fruits were washed to remove dust particle. Fine powder of both dried fruits as well as burned fruit, was pre-

pared. 20 g of fine powder was suspended in 100 ml sterile distilled water, boiled and filtered through Whatman No.1 filter paper and stored at 4°C to be used for further experiments.

Chemical Synthesis of Silver Nanoparticles

The solution of 1mM aqueous AgNO_3 was heated up to boiling. To this solution 5m of trisodium citrate (stabilizing agent) was added drop by drop and mixed vigorously. It was heated up to 2 hours and then cooled at room temperature. It was then centrifuged and pellet (nanoparticles) was dried at 72°C overnight.

Biological Synthesis of Silver Nanoparticles

For biological synthesis, prepared dried and burned fruit extract (10ml) was mixed with 90 ml of 1mM aqueous AgNO_3 separately. Further it was kept at 72°C in water bath. The silver nanoparticle synthesis was identified by the appearance of dark brown color from yellow colored solution.

Characterization of Silver Nanoparticles

a. Ultraviolet-Visible Spectral Analysis

UltraViolet-Visible spectral analysis of reaction mixture was done by using UV-Vis double beam spectrophotometer of Systronics Ltd. within the range of 200-800 nm. Distilled water was used to adjust the baseline.

b. X-Ray Diffraction Analysis

Silver nanoparticle solution obtained was purified by repeated centrifugation at 10,000 rpm for 20 min followed by re-dispersion of the pellet into 10ml of sterile distilled water. After drying of purified silver nanoparticles, the structure and composition were analyzed by XRD. The XRD spectra was recorded using X-ray diffractometer (Bruker GXS D-8) operated at voltage of 40 kV and a current of 30 mA with $\text{CuK}\alpha$ radiation by using 2θ from 10-80°. The crystallite domain size was calculated from the width of the XRD peaks using the Scherrer formula [19].

$$D = 0.94\lambda / \beta \cos \theta$$

Where, D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the Full Width at Half Maximum (FWHM), and θ is the diffraction angle.

c. Topographical Analysis by SEM

Scanning Electron Microscopic (SEM) analysis was done using Hitachi S-4500 SEM machine. Film of the sample were prepared on a carbon coated copper grid by dropping a very small amount of sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

Isolation of Infectious Microorganisms from Burn Skin

The freshly discarded burned patient swab was collected from biomedical waste of Civil Hospital, Ahmednagar. Immediately swab was brought to the lab and Swab culture technique was used to isolate infectious microbes. Swabs were inoculated on Blood agar and

MacConkey's Agar plate and further incubated at 37°C for 24 hr. For further identification, isolated colonies were grown on selective media like Cetrimide agar, Mannitol Salt agar, Worfel-Ferguson agar, Leed's Acinetobacter medium etc. The purity and identification of each isolate was confirmed in laboratory based on morphological and biochemical characterization according to Bergey's manual of determinative bacteriology, 9th edition.

Antibacterial Activity of Silver Nanoparticle

Sterilized Mueller Hinton Agar (MHA) plates were prepared for antibacterial activity determination. Plates were then spreaded with isolated bacterial cultures (*P. aeruginosa*, *S. aureus*, *E. coli*, *Acinetobacter sp.* and *K. pneumoniae*). Sterilized 6 mm cork borer was used to prepare agar wells. Aliquots of 100 µl of chemically and biologically synthesized AgNPs were placed into the wells followed by 10 min incubation in refrigerator. The plates were incubated at 37°C for 24 hrs and zone of inhibition was measured. The results were compared with standard antibiotic Gentamycin (10µg /ml). Assay was performed in triplicates.

Statistical Analysis

The data was analysed using a normal probability plot to confirm that the data followed a normal distribution and the goodness of fit for the data was evaluated using adjusted R-squared. Tukey's Post-hoc analysis was conducted using Tukey's test to identify significant differences in antibacterial activity between the different

bacterial isolates.

Results and Discussion

In the present study, biosynthesis of silver nanoparticles was successfully done using dried and burned fruit powder extract of *Casuarina equisetifolia*. Silver has been known for its antimicrobial capacity against many bacterial strains [18]. Green synthesis of Ag-NPs was done from aqueous silver ions by using the biomass reducing and capping agents of *Casuarina equisetifolia* fruit extract. After 24 hrs the colorless reaction was changed into yellowish brown colour, indicating that formation of AgNPs [16-4]. The plant was previously reported to have properties like antibacterial [20,21], antifungal [21], antioxidant [16], antihyperlipidemic, anti-diabetic [22] and antiulcerogenic [23]. *C. equisetifolia* was reported to have phyto-constituents like β -sitosterol, cholesterol, casuarine, catechin, citrulline, gallicin, gentisic acid, isoquercitrin, juglanin, kaempferol, rutin, trifolin taraxerol, lupenone, lupeol, gallic acid, sitosterol [24,25]. Park, et al., [26] reported that polysaccharides and phytochemicals are usually responsible for green synthesis of nanoparticles. ss

During synthesis period of 24 hours, a characteristic yellowish-brown color was observed on addition of silver nitrate solution to the fruit powder extract, indicating synthesis of AgNPs in biological method (Figure 1).

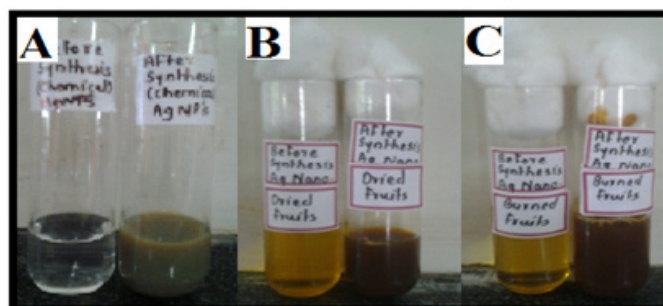


Figure 1: Yellowish brown colloidal solution of silver nanoparticles synthesized using A: chemical method B: Dried fruits and C: Burned fruits.

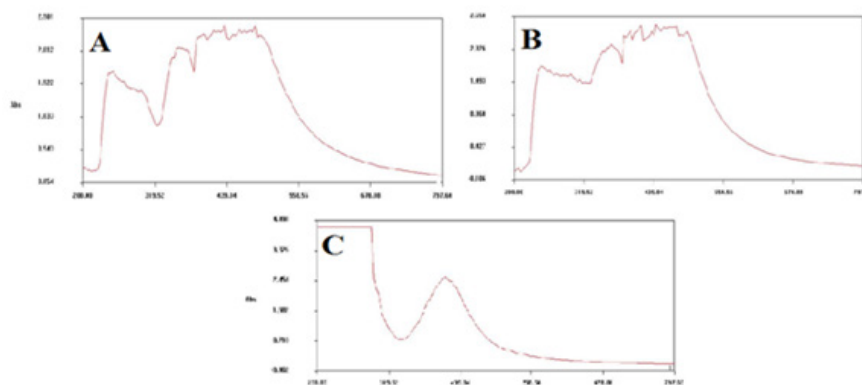


Figure 2: UV-Visible absorption spectra for silver nanoparticles synthesized A: chemically (Peak point-416nm) B: using dried fruits (Peak point-434nm) C: using burned fruits (Peak point-412nm).

Confirmation of AgNP formation was done by taking UV-Vis spectrum of the solution, in the range of 200–800 nm. Result of UV-Vis spectroscopic analysis of these solutions revealed a characteristic absorption peak at 416 nm for chemically synthesized AgNPs, 434 nm and 412 nm for AgNPs synthesized using dried and burned fruits, respectively confirmed the synthesis of AgNPs [1] (Figure 2).

A result of Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD) analysis gives an idea about size, shape and nature of synthesized nanoparticles. Scanning Electron Microscopy (SEM) showed presence of relatively cubic and triangle shaped nanoparticles with average size ranging 1-100 nm, it further confirmed the agglomeration of AgNPs (Figure 3).

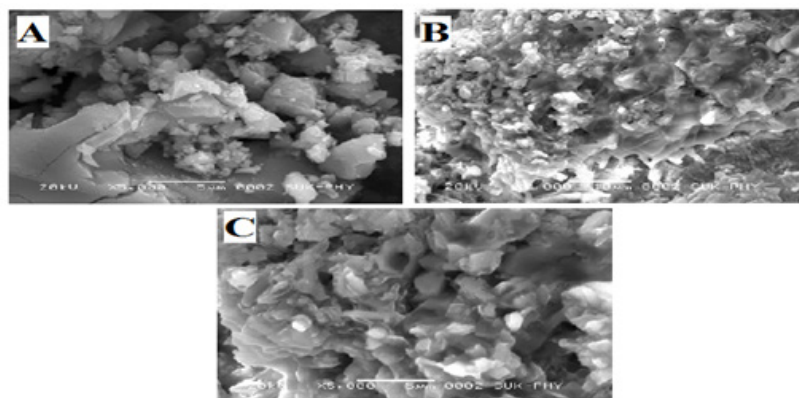


Figure 3: SEM micrograph for silver nanoparticles synthesized by A: Chemical method B: Dried fruits and C: Burned fruits.

X-Ray Diffraction (XRD) analysis revealed crystalline nature of chemically and biologically synthesized AgNPs. Sharp peak in the XRD patterns suggests very small size of nanoparticles. Size of the silver nanoparticles synthesized by chemical method, dried fruits and burned fruits was calculated using Scherrer's formula [19] as 45 nm, 13 nm and 33 nm respectively (Figure 4). This gives resem-

blance with particle size range obtained from results of SEM image. It further indicated that biologically synthesized silver nanoparticles were significantly smaller than chemically synthesized (Figure 3 and 4). It has been reported that small NPs are able to inhibit or destroy microbial cells easily [27].

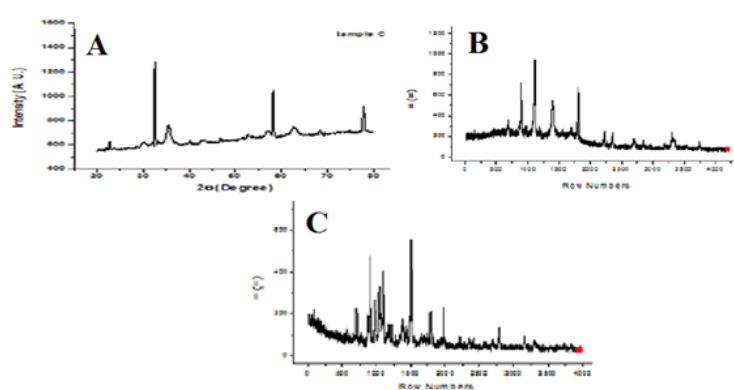


Figure 4: XRD pattern of silver nanoparticles synthesized by A: Chemical method (45nm), B: using dried fruits (13nm) C: using burned fruits (33nm).

Bacteria were isolated from infected burned skin sample on Blood Agar. The colonies giving hemolysis on Blood Agar media were further selected and grown on selective media like Cetrimide agar, Mannitol salt agar, MacConkeys agar, Leed's acinetobacter medium and Worfel-Ferguson medium. Further biochemical characterization was performed for above five isolated colonies. After morphological and biochemical characterization (Table 1), all the

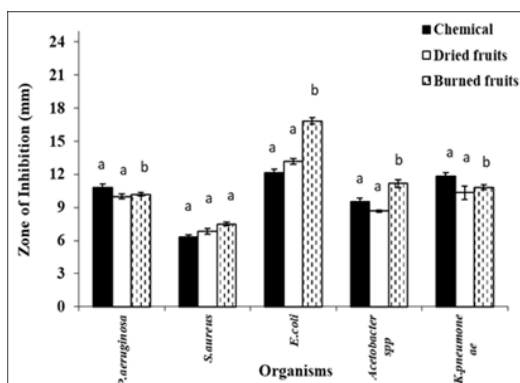
five isolates were identified with the help of Bergey's Manual as Isolate 1- *Pseudomonas aeruginosa*, isolate 2 - *Staphylococcus aureus*, isolate 3 - *Escherichia coli*, isolate 4 - *Acinetobacter sp.* and Isolate 5 - *Klebsiella pneumonia*. These above five isolates were previously reported for infections in burn patients and are found mostly resistant towards different antibiotics used in study [13].

Table 1: Morphological and biochemical characteristics of organisms isolated from burned skin.

Isolates	Gram Char-acter	Motility	Catalase Test	Oxidase Test	Imvic Test			
					Indole	MR	VP	Citrate
1	Gram Negative Rod	+	+	+	-	-	-	+
2	Gram Positive Cocci	-	+	-	-	-	-	+
3	Gram Negative Rod	-	+	-	+	+	-	-
4	Gram Negative Rod	-	+	-	-	-	-	+
5	Gram Negative Rod	-	+	-	-	-	+	+

Bactericidal effect of chemically and biologically synthesized AgNPs was evaluated using agar well diffusion method against previously isolated five pathogenic bacteria. The data was analyzed using a normal probability plot to confirm that the data followed a normal distribution and adjusted R square value is 72.55, indicating a good fit of the model. Tukeys pair-wise comparison clearly indicates that AgNPs synthesised using burned fruit powder of *C. equisetifolia* demonstrated the significant antibacterial activity against all the five isolates (except for *Staphylococcus aureus*) (Figure 5). Nanoparticle size of burned fruit powder was significantly smaller which suggest that tiny NPs may penetrates microbial cell

wall easily and destroy various organelles. It has been observed that AgNPs synthesized from leaves and fruits are effective antimicrobial agent [28]. It was observed that, *E. coli*, *Acinetobacter spp.* is highly susceptible to nanoparticles synthesised using burned fruits of *C. equisetifolia*, whereas *S. aureus* is less susceptible to both chemically and biologically synthesised nanoparticles. In general, AgNPs are nontoxic [29] and used for wound dressing in clinics for many years. The AGNPs are used in wound healing because its takes lesser healing time [30] and show better collagen alignment and greater mechanical strength.

**Figure 5:** Bactericidal effect of Chemically and Biologically synthesized AgNPs.

Conclusion

In present work, we have developed a method for synthesis of AgNPs using fruit of *C. equisetifolia*. To the best of our knowledge, nanoparticles synthesis using burned fruit powder of *C. equisetifolia* has not yet been reported. Biological method gives production of smaller sized silver nanoparticles compare to chemical method. The results of study emphasized on application of synthesized nanoparticle in treatment of skin burn infection. These results clearly indicate that AgNPs could provide alternative topical therapy in treatment of skin burn infection.

Acknowledgement

None.

Conflict of Interest

None.

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