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Research Article

Development of Silver Nanoparticles of Balanites Aegyptiaca Plant Extract for the Effective Treatment of Fungal Diseases

Nishant Prabhaker, Ajay Patel, Umesh Kumar Jain*

Bhopal Institute of Technology and Science–Pharmacy, Bhopal, M.P.

A B S T R A C T

Invasive fungal diseases (IFDs) are an increasingly common complication in critically ill patients in world and are frequently fatal. Because of changes in treatment strategies and the increased use of antifungal prophylaxis, the epidemiology of IFDs has changed substantially in recent years and infections due to Candida species are no longer the majority in many institutions. Invasive candidiasis (IC) can affect individuals with various underlying diseases hospitalized in different parts of hospitals. In recent decades, IC has caused 27–55% mortality in general population. Treatment of invasive fungal infections is challenging as the number of existing antifungals is limited and more problems include: toxicity, drug interactions, and drug resistance. Hence there is need to acknowledge alternative remedies for fungal treatment. Plants are not only important to the millions of people to whom traditional medicine serves as the only opportunity for health care and to those who use plants for various purposes in their daily lives, but also as a source of new pharmaceuticals. The use of silver nanoparticles (AgNPs) against various pathogens is now being well recognized in the agriculture and health sector.

Keyword: Epidemiology, Nanoparticles, Plant Extract, Fungal Diseases

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*Address for Correspondence:

Dr. Umesh Kumar Jain, Bhopal Institute of Technology and Science – Pharmacy, Bangrasia, Bhojpur Road, Bhopal, M.P., 462047

INTRODUCTION:

Nanotechnology is emerging as a rapidly growing field with its application in Science and Technology for the purpose of manufacturing new materials at the nanoscale level [1]. The word “nano” is used to indicate one billionth of a meter or 10−9. The term Nanotechnology was coined by Professor Norio Taniguchi of Tokyo Science University in the year 1974 to describe precision manufacturing of materials at the nanometer level [2]. The concept of Nanotechnology was given by physicist Professor Richard P. Feynman in his lecture There’s plenty of room at the Bottom [3]. Nanoparticles are clusters of atoms in the size range of 1–100 nm. “Nano” is a Greek word synonymous to dwarf meaning extremely small. The use of nanoparticles is gaining impetus in the present century as they possess defined chemical, optical and mechanical properties. The metallic nanoparticles are most promising as they show good antibacterial properties due to their large surface area to volume ratio, which is coming up as the current interest in the researchers due to the growing microbial resistance against metal ions, antibiotics and the development of resistant strains [4]. Bionanotechnology has emerged up as integration between biotechnology and nanotechnology for developing biosynthetic and environmental-friendly technology for synthesis of nanomaterials. Different types of nanomaterials like copper, zinc, titanium, magnesium, gold, alginate and silver have come up but silver nanoparticles have proved to be most effective as it has good antimicrobial efficacy against bacteria, viruses and other eukaryotic micro-organisms. Silver nanoparticles used as drug disinfectant have some risks as the exposure to silver can cause agyrosis and argyria also; it is

toxic to mammalian cells. For centuries silver has been in use for the treatment of burns and chronic wounds. As early as 1000 B.C. silver was used to make water potable [5]. Silver nitrate was used in its solid form and was known by different terms like, "Lunar caustic" in English, "Lapis infernale" in Latin and "Pierre infernale" in French. In 1700, silver nitrate was used for the treatment of venereal diseases, fistulae from salivary glands, and bone and perianal abscesses. In the 19th century granulation tissues were removed using silver nitrate to allow epithelization and promote crust formation on the surface of wounds. Varying concentrations of silver nitrate was used to treat fresh burns [6]. In 1881, Carl S.F. Crede cured opthalmia neonatorum using silver nitrate eye drops. Crede's son, B. Crede designed silver impregnated dressings for skin grafting. In the 1940s, after penicillin was introduced the use of silver for the treatment of bacterial infections minimized [7]. The antimicrobial property of silver is related to the amount of silver and the rate of silver released. Silver in its metallic state is inert but it reacts with the moisture in the skin and the fluid of the wound and gets ionized. The ionized silver is highly reactive, as it binds to tissue proteins and brings structural changes in the bacterial cell wall and nuclear membrane leading to cell distortion and death. Silver also binds to bacterial DNA and RNA by denaturing and inhibits bacterial replication [8]. The silver nanoparticles show efficient antimicrobial property compared to other salts due to their extremely large surface area, which provides better contact with microorganisms. The nanoparticles get attached to the cell membrane and also penetrate inside the bacteria. The bacterial membrane contains sulfur-containing proteins and the silver nanoparticles interact with these proteins in the cell as well as with the phosphorus containing compounds like DNA. When silver nanoparticles enter the bacterial cell it forms a low molecular weight region in the center of the bacteria to which the bacteria conglomerates thus, protecting the DNA from the silver ions. The nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death. The nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity [9]. Balanites aegyptiaca underutilized tree species are good sources of food, fodder and possible therapeutic agents. Balanites aegyptiaca (L.) Delile belongs to the Zygophyllaceae family and is popularly known as "desert date", reflecting its edible fruits. Various parts of the plant are used in Ayurvedic and other folk medicines for the treatment of different ailments such assyphilis, jaundice, liver and spleen problems, epilepsy, yellow fever and the plant also has insecticidal, antihelminthic, antifeedant, molluscicidal and contraceptive activities [10]. Flowers are small, inconspicuous, hermaphroditic, and pollinated by insects. Seeds are dispersed by ingestion by birds and animals. The tree begins to flower and fruit at 5 to 7 years of age and maximum seed production is when the trees are 15 to 25 years old [11]. Nanoparticles have been shown to exhibit various novel properties and these properties, on other hand, rely upon the size, shape, and morphology of these particles. Moreover, these physical characteristics enable them to interact with microbes, plants, and animals. Smaller- sized particles have shown more toxicity than larger-sized nanoparticles. AgNPs have shown growth inhibition of many fungi like Aspergillus fumigates, A. niger, A. flavus, Trichophyton rubrum, Candida albicans, and Penicillium species. Therefore, this investigation is focused on the formulation and evaluation of silver nanoparticles of Balanites aegyptiaca plant extract (flower) for the effective treatment of fungal diseases.

Material and methods:

All chemicals and solvents were of analytical mark (AR Grade) and were procured from Sigma Aldrich, Ranbaxy fine chemicals Ltd., LOBA chemicals Ltd., S.D. fine chemicals Ltd., Spectrochem chemicals. Pre-coated TLC plates having silica gel were purchased from Merck. All the solvents used for HPLC study were procured from JT Baker and Fischer scientific Ltd.

Plant material:

The particular plant objects naming *Balanites Aegyptiaca* flower was collected in the month of May from the Bhopal region of (India). This is a logical advance method, which justify for authentication and resolve of character, transparency and value of a crude drug. This exhaustive and efficient pharmacognostic reading provides precious knowledge for potential investigation.

Macroscopic studies:

The selected crude drugs were used to learn organoleptic characters as, color, odour, appearance, taste, texture etc. Primary, the strength of odor was determined (none, weak, distinct, strong) followed by odor sensation (aromatic, fruity, musty, moldy, rancid, etc.) was studied. Taste was specifically classified as aromatic, pungent, sweet, sour, astringent, mucilaginous, or bitter. The roots were brown in color and surface was irregular and rigid due to extent rotten of longitudinal striation. Internal wall of root was creamy white, soft and distorted.

Physicochemical Evaluation:

Physiochemical values for chosen plant sedate were distinguished by the official techniques. The different parameters incorporate, for example, debris esteem and extractive qualities were resolved. The WHO rules on quality control strategies for restorative plant materials was streams for assessing the above parameters. Debris esteems (Total debris, Acid insoluble debris and Water solvent debris), Extractive qualities, Loss on drying and pH were resolved for *Balanites Aegyptiaca* bloom. The accompanying physicochemical examination was resolved for the powder medicate.

Synthesis of Silver Nanoparticles:

0.1 M of aqueous solution of silver nitrate (AgNO3) was prepared and used for the synthesis of silver nanoparticles. 5 mL of flower extract of was added to 45 mL of 0.1 M AgNO3 solution for bioreduction process at room temperature. AgNPs were synthesized by addition of aqueous *Balanites Aegyptiaca* plant extract to aqueous silver nitrate solution. Varying volume ratios (10:1, 10:2, 10:3, and 10:4) of silver nitrate solution to quantity of plant extract were optimized to yield desired AgNPs.

Purification of AgNPs:

Refrigerated high speed centrifuge (Kubota 6500, Japan) was used to purify synthesized AgNPs. Biomatrix consisting of AgNPs and extraneous plant extract were subjected to centrifuge at 17000 rpm, 4°C for 20 minutes. After centrifugation, settled AgNPs were isolated and residual supernatant remaining after centrifuge (RSL) was stored independently for in vitro antioxidant assay. UV-Visible spectrum was verified before and after centrifugation to endorse the purity of synthesized AgNPs. Purified AgNPs were freeze dried (lyophilized) and stored for characterization.

Characterization of AgNPs:

Dynamic light scattering studies: Zetasizer (Malvern, UK) was used to examine the zeta potential and particle size characterstics of colloidal dispersion of AgNPs. Prior to analysis, AgNPs were diluted using deionized water.

Fourier transform infrared spectroscopy (FT-IR): FT-IR spectra of lyophilized AgNPs was examined by using FT-IR (Shimadzu, Japan). Samples were investigated by KBr pellet method in the range 450 4000 cm at resolution of 4 cm.

UV-Visible Absorbance Spectroscopy: UV-Visible spectroscopy analysis was carried out on a Systronic UV-Visible absorption spectrophotometer 1800 with a resolution of ±1 nm between 200 and 1000 nm processing a scanning speed of 200 nm/min. Equal amounts of the suspension (0.5 mL) were taken and analysed at room temperature. The progress of the reaction between metal ions and the leaf extract was monitored by UV-Visible spectra of silver nanoparticles in aqueous solution with different wavelength in nanometers from 340 to 800 nm. The reduction of silver ions and formation of silver nanoparticles occurred within an hour of reaction. Control was maintained by using AgNO3.

Antifungal activity of AgNPs: The antifungal activity of AgNPs was evaluated using agar well diffusion against Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Staphylococcus aureus. All tested fungal strains were grown on PDA plates and incubated for 3–5 days at 30 °C. The fungal suspension was prepared in sterilized phosphate buffer solution (PBS) pH 7.0, and then the inoculum was adjusted to 107 spores/mL after counting in a cell counter chamber. One milliliter was uniformly distributed on agar MEA plates. Using a sterile cork-borer, wells (8 mm) were cut; 100 µL of AgNPs and AgNO3 were transferred to each well individually and left for 2 h at 4 °C. Nystatin was used as a standard antifungal, and then the plates were incubated for 3 days at 30 °C. After incubation, the inhibition zones were determined and recorded. Moreover, different concentrations of AgNPs were evaluated as antifungal to detect the minimum inhibitory concentration (MIC).

RESULT AND DISCUSSION

Plant material *Balanites Aegyptiaca* flower: Flowers found as solitary or in group of two to five. Calyx is campanulate, three lobes (4 x 3 cm). Flower has glabrous outside and silky inside touch. It has five petals elliptic-obovate, fleshy. Flowers have 65 – 80 stamens having 3 to 7.5 cm length. It has conical ovary having many ovules. Flower style exceeding the stamens. Flowers are acrid and bitter in taste and having characteristic taste. Physicochemical parameter such as Ash values (Total ash, Acid insoluble ash and Water-soluble ash), Extractive values and Loss on drying selected plant drugs were performed. Ash values of crude drug provide an idea about the inorganic composition or earthy matter and other impurities present in drug. The extractive values are mainly useful for the determination of adulterated or exhausted drug. All parameters of selected drugs found within the limit as per API. Results exposed that %w/w yield in successive extraction of *Balanites Aegyptiaca* flower in petroleum ether, chloroform, methanol and water were 3.5, 1.7, 7.4 and 10.1 respectively indicating higher in water and low in chloroform. Colour of extract of *Balanites Aegyptiaca* flower was Yellowish orange sticky in petroleum ether, Light red in chloroform, dark orange in methanol and dark red in water. Consistencies of the flower extracts were sticky and semisolid in petroleum ether while non sticky and solid in chloroform, methanol and water extracts. Dissimilar result of %w/w yield, colour and consistency of successive extracts of *Balanites Aegyptiaca* flower helps in identification of plant

Phytochemical studies of extract from *Balanites Aegyptiaca* flower: Qualitative phytochemical examination of successive extracts in petroleum ether, chloroform, methanol and water of *Balanites Aegyptiaca* flower powder was carried out and outcomes are shown in **Table 1.** The results indicated that the alkaloids and flavonoid were present in chloroform, methanol extracts of *Balanites Aegyptiaca* flower. Saponin, proteins, tannins and carbohydrates present in methanol and water extract. Phyosterols and triterpenes are present in pet ether and chloroform extracts. Cardiac glycoside and coumarins are absent in all extracts.

Table 1: Phytochemical analysis of *Balanites Aegyptiaca* flower extracts

Tests of Phytoconstituents	Methanol extract
1. Alkaloids	
a) Mayer's reagent	+
b) Dragendorff's reagent	+
2. Flavonoids	
a) Shinoda test	+
3. Saponins	
a) Froth test	+
4. Carbohydrate	
a) Molisch's test	+
c) Test for gums	+
d) Test for mucilage	+
5. Phytosterols	
a) Libermann-Burchard test	-
c) Salkowski reaction:	-
6. Tannins and Phenolic	
a) With Lead acetate	+
7. Cardiac glycoside	
a) Legal's test	-
b) Keller killiani test	-
8. Coumarins	
a) With ammonia	-
b) Hydroxylamine HCl	-
9. **Proteins**	
a) Biuret test	+
10. Triterpens	
a) Vanillin sulphuric acid	-

Characterization of AgNPs:

Dynamic light scattering studies: Hydrodynamic Particle size, zeta potential and PDI (polydispersity index) results of synthesized AgNPs is summarized in table 6.1. Plant extract mediated synthesized AgNPs at room temperature were found to have least particle size and improved zeta potential when compared with AgNPs synthesized by stirring and heating. Alkaline condition although was favorable and lead to the formation of AgNPs with smaller particle size but lower magnitude of zeta potential lead to sedimentation of particles.

Fourier transforms infrared spectroscopy (FT-IR): Lyophilized AgNPs were analyzed by FT-IR spectrophotometer to evaluate the presence of phytochemicals coating onto silver nanoparticles. FT-IR spectrum of plant extract mediated synthesized AgNPs showed prominent peaks at 3637 cm-1, 3429 cm-1, 3313 cm-1, 3178 cm-1, 2926 cm-^{1}, 1728 cm-1, 1614 cm-1, 1585 cm-1, 1317 cm-1 and 1191 cm-1 as shown in Figure 1.

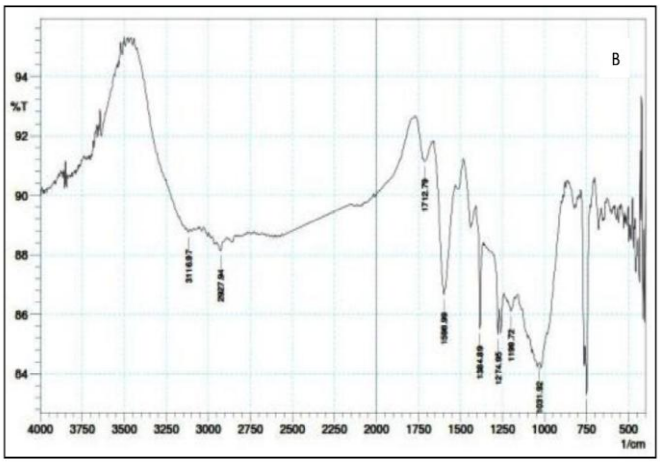

Figure 1: FT-IR spectrum of plant extract mediated synthesis of AgNPs at room temperature

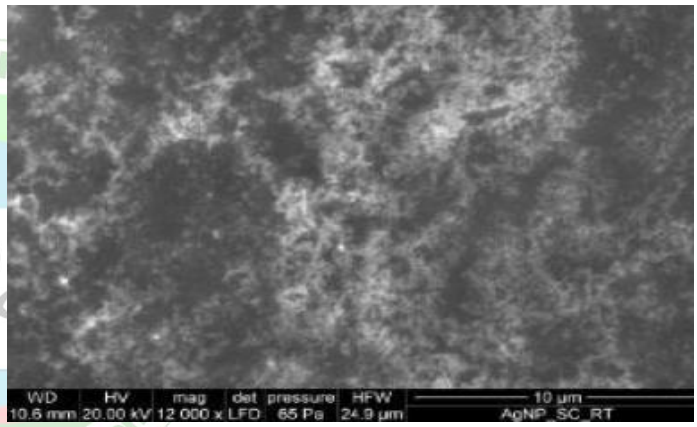

Figure 2: SEM image of plant extract mediated synthesis of AgNPs at room temperature

Antifungal Activity:

In this study, the antifungal activity of AgNPs toward Aspergillus species was evaluated using the agar well diffusion method (Figure 6.7). Results illustrated that biosynthesized AgNPs exhibited promising antifungal activity against all tested fungal strains. AgNPs had the ability to inhibit fungal growth of Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi and Staphylococcus aureus at concentrations of 500 µg/mL where inhibition zones were 16, 20, 26, and 19 mm, respectively. Moreover, MICs of AgNPs against Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi and Staphylococcus aureus were determined as illustrated in Table 6.2 MIC results showed that A. flavus was the most sensitive fungus to AgNPs, which inhibited growth at concentrations of 15.6 µg/mL, while for the other fungal species, inhibition of growth was observed at 125 µg/ mL for A. niger and 62.5 µg/mL for A. terreus and A. fumigatus. AgNPs in solution at higher concentrations may be able to adhere to and saturate fungus hyphae, destroying the fungus cells. Such an inhibitory effect can be attributed to Ag+ that

primarily affects the function of membrane-associated enzymes such as those found in the respiratory chain. Ag+ may also affect the expression of some microbial proteins and enzymes. Disruption of DNA replication has also been documented. AgNPs can also interact with substrates through a process known as competitive inhibition, inactivating the enzymes and preventing the production of products required for cell activity.

SUMMARY AND CONCLUSION:

Preliminary phytochemicals investigation of aqueous extract was performed revealed the presence of flavonoids, polyphenols, carbohydrates which are responsible for synthesis of AgNPs and proteins responsible for its capping and stabilization. Presence of these phytochemicals and reported antifungal activity served as a base for use of the plants as green source of synthesis of AgNPs. Aqueous plant extract was added to aqueous solution of silver nitrate leading to the formation of brown colored AgNPs. Green synthesis of AgNPs by using plant extract is very unique as it yields nanoparticles of different stability and particle characteristics depending on the phytochemicals of the plant extract used in the synthesis. On addition of aqueous plant extract in varying ratios to silver nitrate solution, there was initial visual change from colorless to dark brown which suggested the formation of AgNPs. UV-Visible spectroscopic analysis of AgNPs colloidal dispersion showed distinct peak at 436 nm (characteristic peak of AgNPs) confirmed the synthesis of AgNPs. Although varying ratios of plant extract to silver nitrate solution (10:1. 10:2 and 10:3) demonstrated the color change from colorless to dark brown within 2 h but10:1 ratio was finalized where least amount of plant extract was utilized for synthesis of AgNPs. The reason for formation of AgNPs could be attributed to presence of polyphenols and flavonoids in aqueous plant extract for reducing silver nitrate to AgNPs and proteins for stability of AgNPs. Application of stirring and heating enhances the rate of reaction and nanoparticles were formed in less time when compared to room temperature condition. Stirring and heating leads to decrease in time from 2 hours to 30 minutes for formation AgNPs. In case of alkaline condition brown color was formed immediately after addition of plant extract. Resultant AgNPs obtained were purified by centrifugation and characterized by using UV-Visible spectroscopy.

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