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METHODOLOGICAL ADVANCEMENTS IN GREEN NANOTECHNOLOGY AND THEIR APPLICATIONS IN BIOLOGICAL SYNTHESIS OF HERBAL NANOPARTICLES

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Abstract: The recent technological advancement in nanotechnology has opened new avenues for research and development in the field of herbal and medicinal plants biology. Development of reliable and eco-friendly processes for synthesis of nanoparticles is an important step for introduction of applications of nanotechnology into herbal research. In the past decade, there has been much concern about the methodological advancement of technology for synthesis and characterization of herbal and medicinal plants mediated nanoparticles. The present article provides a comprehensive review on the recent advances brought into methodology for biological and eco-friendly synthesis and characterization of herbal and medicinal plants mediated nanoparticles.

Keywords: Biological Synthesis, Gold and Silver Nanoparticles, Green Nanotechnology, Herbal and Medicinal Plants, Methodological Advancement, Synthesis and Characterization.

INTRODUCTION

Considerable efforts are being made on the global level to develop and implement eco-friendly technologies for production of herbal based consumer products for providing better healthcare solutions. Nanoparticles are considered as the fundamental building blocks of nanotechnology (1, 2). The synthesis and characterization of nanoparticles and their applications represent a rapidly growing concept and an emerging trend in science and technology (3, 4). In the recent years, there has been much concern about the synthesis of environment-friendly nanoparticles that do not produce toxic wastes in their process of synthesis (1). This can only be achieved through benign synthesis processes of biological nature which are considered safe and ecologically sound for nanomaterial fabrication as an alternative to conventional physical and chemical methods (5). In several studies, the biological routes to the synthesis of nanoparticles have been proposed by exploiting microorganisms and vascular plants (6-10). However, the utilization of herbal and medicinal plant extracts for the synthesis of nanoparticles is a relatively recent activity. In a number of studies, gold and silver have been used mostly for the synthesis of stable dispersions of nanoparticles using the extracts of herbal and medicinal plants (11-15).

The silver nanoparticles have some advantage over other nano-forms because silver nanoparticles are reported to be nontoxic to human and most effective against bacteria, viruses, and other eukaryotic micro-organisms at very low concentration and without any known side effects (16). In the recent years, much advancement is brought to the technology for synthesis and characterization of nanoparticles. The current review of methodology provides the technological advancement for biological and eco-friendly synthesis and characterization of herbal and medicinal plants mediated nanoparticles.

Biological synthesis of herbal and medicinal plants mediated nanoparticles:

Extraction of phytochemicals: Several studies have reported the biological extraction of phytochemicals. Linga Rao et al (17) have reported the biological synthesis of silver nanoparticles using *Svensonia hyderabadensis* leaf extract. In their study, they collected the fresh and healthy leaves of *S. hyderabadensis*, a member of Verbenaceae family. The leaves were washed, cleaned and pressed with blotting paper, dried in shade and grinded to make the fine powder. The 5g of powder was taken into 250ml conical flask and 100 ml of distilled water was added to it. The mixture was set for boiling at 100°C for 10min. The prepared leaf extract was separated by standard filtration methods.

Zargar et al (18) have reported the green synthesis of silver nanoparticles using *Vitex negundo* L. The leaves were washed and dried in an oven dryer at 40°C for 48 hours, grinded to powder, stored in dark glass bottles and kept at -20°C for further studies. 20 g of this powder was extracted with methanol (1:10 w/v) overnight at 40°C using a shaking water bath. After filtration with Whatmann filter paper no. 1 using vacuum pump, the residue was re-extracted. The solvent was completely removed using a rotary vacuum evaporator at 40°C. The concentrated extract was kept in dark bottles at 4°C until used.

Another study was carried out by Masurkar et al (19), who reported rapid biosynthesis of silver nanoparticles using *Cymbopogan citratus* (Lemongrass). About 50 g of fresh leaves of *C. citratus* were washed thoroughly, cut into fine pieces, dipped into a beaker containing 200ml of distilled water and boiled for 10-12min. The prepared extract was filtered through Whatmann filter paper and stored for further studies.

Devi et al (20) reported the antimicrobial efficacy of green synthesized silver nanoparticles from the medicinal plant *Plectranthus amboinicus*. Fresh plant material of *P. amboinicus* was collected and the aqueous extract of sample was prepared using the freshly collected leaves (25g), by washing in running tap water and then in distilled water, followed by boiling in 100 ml of distilled water at 60°C for about 5 min. Then the extract was filtered through gauze cloth and used for further experiments.

Prasad et al (21) reported the biogenic synthesis of silver nanoparticles using *Nicotiana tobaccum* leaf extract. In this study, about 5g leaves of *N. tobaccum* were collected and washed thoroughly. The leaves were cut into fine pieces and subsequently macerated in 20ml buffer of Tris-HCl of pH 8.0 with the help of mortar and pestle. The thick slurry thus recovered was then subjected to centrifugation at 10,000 rpm for 5min at 4°C. The supernatant obtained was transferred into sterile centrifuge tubes for preservation in refrigerated condition for using as a precursor for synthesis of silver nanoparticles.

Prasanth et al (22) reported synthesis of silver nanoparticles using extracts of eighteen medicinal herbs, named as Maramanjal, Koduveli, Vasambu, Vilangam, Vepampattai, Jeragam, Mahali, Vendhiyam, Kudagapalari, Devadaru, Pavu, Athimaduram, Dhaniya, Cherrupunneri, Vetiver, Karuncheeragam, Gugulu, and Karpokarasi. The herbs were washed, dried over water absorbent paper and cut into small pieces and dispensed in 100 ml of sterile distilled water and boiled for 1h at 80°C. The herbal extracts were collected in separate conical flasks by standard filtration method.

Thirumurugan et al (23) have reported the biotechnological synthesis of gold nanoparticles of *Azadirachta indica* leaf extract. In the study, the leaves of *A. indica* were collected and allowed to dry for 2 weeks at room temperature. The plant leaf broth solution was prepared by taking 5g of thoroughly washed and finely cut leaves in a 300ml Erlenmeyer flask with 100ml of sterile distilled water. The mixture was set for boiling for 5min. The solution thus prepared was filtered and stored at 4°C and used within a week.

Singh et al (24) have reported the green biogenic approach for synthesis of gold and silver nanoparticles using *Zingiber officinale*. The extract was prepared by taking 10 gm thoroughly washed ginger rhizome, taking out the upper part by the knife, chopped into the fine pieces and converting it into the paste using mortar-pestle followed by addition of 40ml millipore water, boiling for 2 min followed by filtering. The solution was centrifuged at 5000 rpm for 15min. The supernatant was collected which was further used as ginger rhizome broth.

Annamalai et al (25) reported the biosynthesis and characterization of silver and gold nanoparticles using aqueous leaf extraction of *Phyllanthus amarus* Schum. & Thonn. The fresh leaves of *P. amarus* were washed several times with ultra pure water to remove dust. Leaf extract used was prepared from 5g of thoroughly washed leaves which were heated in 50ml ultrapure water for 5min in an Erlenmeyer flask using a water bath. The filtered leaf extract was stored at -15°C for further use, being usable for one week.

Raghunandan et al (26) reported the biosynthesis of stable polyshaped gold nanoparticles from microwave-exposed aqueous extracellular anti-malignant guava (*Psidium guajava*) leaf extract. For preparing the aqueous extracellular solution of guava leaves (*P. guajava*) for biosynthesis, 10 g of freshly collected guava leaves cut into appropriate size (~5 cm×1 cm) were taken into a 250ml wide-neck Borosil conical flask and washed several times with deionized water. Approx 200ml deionized water was added to the flask containing washed, freshly cut guava leaves and exposed to microwave for 3min. The resultant crude extract was filtered with Whatmann filter paper no. 40 and stored for further use.

Balaprasad Ankamwar (27) reported the biosynthesis of gold nanoparticles (green-gold) using leaf extract of *Terminalia catappa.* He took 10 g of thoroughly washed and finely cut *T. catappa* leaves in a 500 ml Erlenmeyer flask with 40ml of sterile distilled water and boiled it for 15 min. The solution was filtered and stored for further studies.

Synthesis of nanoparticles: For the synthesis of silver nanoparticles, 1mM silver nitrate ($AgNO_3$) solution was prepared and stored for further use. 5ml of plant extract (5%) was taken into conical flask and to this 50ml of 1mM $AgNO_3$ solution was added drop wise with constant stirring at 50-60°C and observed the color change (17-22). The color change of the solution was checked periodically. The conical flask was incubated at room temperature for 48 hours. The change in color of the solution to the dark brown indicates the synthesis of silver nanoparticles from the plant extract. This content was centrifuged at 10,000 rpm for 15min. The supernatant was used for the characteristics of the silver nanoparticles through characterization techniques.

For the synthesis of gold nanoparticles, 1mM of chloroauric acid ($HAuCl_4$) was reduced using 50 ml of 5% plant extract at room temperature resulting in a dark ruby pink-red solution indicating the formation of gold nanoparticles (23-27).

Characterization of nanoparticles:

Several techniques have been reported worldwide for the characterization of herbal and medicinal plants based nanoparticles.

Ultra Violet- Visible (UV-Vis) spectrophotometry: A number of studies have reported monitoring of bio-reduction of silver ions in aqueous solution by UV-Vis spectrophotometer (17-22, 28-32). According to these studies, reduction of pure Ag^+ ions was usually monitored after 3 to 5 hours of diluting the small aliquot of the sample into distilled water (17, 20).

Fourier Transform-Infra Red (FT-IR) spectroscopy: An infrared spectrum (IR) represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same IR. Therefore, IR results in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, IR is an excellent tool for quantitative analysis. Because all of the frequencies are measured simultaneously, most measurements by FT-IR are made in a matter of seconds rather than several minutes.

Several investigators have reported FT-IR analyses of herbal and medicinal plant mediated silver nanoparticles (21, 29, 32-35). According to a study carried out by K. Mallikarjuna *et al*. (34) the bio-reduced silver nitrate solution was centrifuged at 10,000 rpm for 15 min and the dried samples were grinded with KBr pellets used for FT-IR measurements. Other studies suggest the freezing of the leaf extracts containing nanoparticles using a lyophilizer (29, 35). The powder obtained in this manner was then subjected to FT-IR analysis.

Transmission Electron Microscopy (TEM): TEM is commonly used for imaging and analytical characterization of the nanoparticles to assess the shape, size, and morphology (18, 19). The outstanding resolution achieved by TEM is an excellent fit for these extremely challenging studies (21). According to several studies carried out by different investigators, thin films of the samples were prepared on carbon coated copper grids by dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper. The films thus prepared on the TEM grid were then allowed to dry under a mercury lamp for 5 min (28, 32, 34).

Scanning Electron Microscopy (SEM): The SEM uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, crystalline structure and orientation of materials making up the sample. In most applications, data are collected over a selected area of the surface of the sample, and a two-dimensional image is generated that displays spatial variations in these properties. For this purpose, thin films of the samples were prepared by the investigators on carbon coated copper grids by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and the films on the SEM grid were allowed to dry under a mercury lamp for 5 min (17, 20, 22, 29).

Atomic Force Microscopy (AFM): AFM is an important biophysical technique for studying the morphology of nanoparticles and bio-molecules. Using the AFM, individual particles and groups of particles can be resolved. Software-based image processing of AFM data can generate quantitative information from individual nanoparticles and between groups of nanoparticles. For individual particles, size information (length, width, and height) and other physical properties (such as morphology and surface texture) can be measured. AFM can be performed both in liquid or gas mediums. This capability can be very advantageous for nanoparticle characterization. AFM has several advantages over SEM and TEM for characterizing nanoparticles. Images from an AFM represent data in three dimensions, so that it is possible to measure the height of the nanoparticles quantitatively (36, 37). According to Satyavani *et al*. (35) a small volume of sample was spread on a glass cover slip

surface mounted on the AFM stub and was dried with nitrogen flow at room temperature. Minimum of five images for each sample were obtained with AFM and analyzed to ensure reproducible results.

X-Ray Diffraction (XRD): XRD is a versatile, non-destructive analytical method for identification and quantitative determination of various crystalline forms. According to studies, the solution of silver nanoparticles obtained was purified by repeated centrifugation at 10,000 rpm for 20 min followed by re-dispersion of the pellet of silver nanoparticles into 10 ml of distilled water. After freeze drying of the purified silver particles, the structure and composition of silver nanoparticles were analyzed by XRD (17, 18, 22, 29, 34). The diffraction occurs as waves interact with a regular structure whose repeat distance is about the same as the wavelength. The phenomenon is common in the natural world and occurs across a broad range of scales.

Energy Dispersive X-Ray Spectra (EDX/EDS): EDS is an analytical technique which utilizes x-rays that are emitted from the specimen when bombarded by the electron beam to identify the elemental composition of the specimen. When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms on the surface of specimen. A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons. The EDS X-ray detector measures the number of emitted X-rays versus their energy. The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the energy versus relative counts of the detected X-rays is obtained and evaluated for qualitative and quantitative determinations of the elements (38). According to studies conducted by a few investigators, a drop of the solution was placed on carbon coated copper grid and exposed to infrared light for 45min for performing EDX analysis of the nanoparticles (19, 21, 33).

Dynamic Light Scattering (DLS): DLS is one of the most popular techniques which is used to determine the size of particles. Shining a monochromatic light beam, such as 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 incoming light. This change is related to the size of the particle. Using DLS, it is possible to compute the sphere size distribution and give a description of the particle's motion in the medium measuring the diffusion coefficient of the particle by using autocorrelation function (28).

Antimicrobial Assays: The antibacterial assays have been carried out on various pathogenic bacteria like, *Escherichia coli* (18, 19, 21, 22, 28-30, 35), *Pseudomonas aeruginosa* (17, 21, 35), *Pseudomonas aureus* (19, 30), *Pseudomonas vulgaris* (35), *Staphylococcus aureus* (18, 22, 29, 35), *Staphylococcus pyrogens* (29), *Bacillus subtilis* (21, 29, 30), *Salmonella typhimurium* (19, 28, 29), *Klebsiella pneumoneae* (19, 30) and *Micrococcus spp.* (22) by standard disc diffusion method on Luria Bertani (LB) agar media. According to these studies, the bacterial strains were inoculated into LB media followed by their incubation at 37°C for 12h. Inoculums of 100µl were drawn from overnight grown culture and spread evenly over the surface of LB agar plate. Sterile discs of six millimeter width were dipped into plant extract containing 17mg/l of $AgNO_3$. Dried discs were placed over culture plates followed by an incubation of 12 h at 37°C. The antibacterial activity was assigned by measuring the inhibition zone formed around the discs (21).

In another study, the nanoparticles were tested for antibacterial activity by agar well-diffusion method against pathogenic bacteria *P. aeruginosa*, and *S. aureus*. The pure cultures of bacterial pathogens were subcultured on nutrient agar. Wells of 10 mm diameter were made on nutrient agar plates using gel puncture. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Using a micropipette, different concentrations of the samples of nanoparticle solution (10µl, 20µl and 50µl) were poured into each well on all plates. After incubation at 37°C for 24 hours, the different levels of zones of inhibition of bacteria were measured (39).

The nanoparticles have also been tested for antifungal activity by agar well-diffusion method against pathogenic fungi (*A. niger* and *A. flavus*). The pathogens were subcultured on PDA media. Wells of 10 mm diameter were made on PDA plates using gel puncture. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Using a micropipette, different concentrations of the samples of nanoparticle solution (10µl, 20µl and 50µl) were poured into each well on all plates. The plates were kept at room temperature for 48 h and the clear zones were measured (39).

As well as, the antifungal assays have been carried out for *Asprgillus niger* (17, 20, 22, 30), *A. flavus* (20, 30), *A. fumigatus* (20), *Fusarium oxysporum* (17, 22, 30), *Curvularia lunata* (17, 30), *Candida albicans* (19, 20, 22), *Rhizoctonia solani* (22), *Alternaria alternata* (22), and *Rhizopus arrhizus* (17, 30) on potato dextrose agar (PDA) and potato dextrose broth (PDB) media. According to disc diffusion method, the fungal strains were cultured on PDA media followed by their inoculation in PDB media. Inoculums of 100µl were drawn from the grown culture and spread evenly over the surface of PDA agar plate. The dried discs were placed over culture plates followed by an incubation of 72h at 28°C. The antifungal activity was assigned by measuring the inhibition zone formed around the discs (21, 40).

CONCLUSION

Several plant extracts have been successfully used for extracellular synthesis of silver and gold nanoparticles. Some modern analytical techniques and antimicrobial assays have been applied to characterize the nanoparticle morphology. The applications of such eco-friendly nanoparticles in bactericidal, wound healing and other medical and electronic applications make the biological approach potentially exciting for the large- scale synthesis of other nanoparticles. From a technological point of view, the silver and gold nanoparticles have potential applications in biomedical fields, agriculture, foods and herbal research among several others. The biological procedures have considerable advantages such as cost-effectiveness, compatibility for medical and pharmaceutical applications as well as large scale commercial production. In future, it would be significant to understand the clear mechanism of biosynthesis and to technologically engineer the nanoparticles in order to achieve better control over size, shape, and absolute monodispersivity which would further enhance the applications of nanoparticles in the related fields.

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