Assessment of antibacterial and antifungal activities of silver nanoparticles obtained from the callus extracts (stem and leaf) of *Tridax procumbens* L.

Himakshi Bhati-Kushwaha and C P Malik*
School of Life Sciences, Jaipur National University, Jaipur 302 017, India

Received 14 March 2013; revised 28 May 2013; accepted 14 June 2013

Now-a-days metal nanoparticles are potentially used as tools in the field of engineering and biological sciences as well as in industries and communication technologies. The important aspect of nanotechnology is to develop dependable, environmentally benign processes for the synthesis of nanoscale materials. Presently, we describe a cost-effective and eco-friendly technique for green synthesis of biogenic nanoparticles from the extract of callus obtained through stem and leaves of *Tridax procumbens* L., which acted as a reducing as well as capping agent. This plant has been opted for the present study for its known medicinal properties. This method of synthesizing nanoparticles led to rapid reduction of silver ions, resulting in the formation of stable crystalline nanoparticles in the solution. The synthesis of biogenic nanoparticles was carried out by adding silver nitrate solution to the callus extract. Nanoparticles were screened using UV-Visible absorption spectroscopy and were well characterized by SEM, XRD and FTIR analysis. The process responsible for the formation of nanoparticles from the biomass of plant callus is called bio-mineralization. The synthesized biogenic nanoparticles were further tested for antimicrobial activity using *Escherichia coli*, *Vibrio cholerae*, *Aspergillus niger* and *A. flavus*. Later, the activities of synthesized biogenic nanoparticles were also compared with various plant extracts (aqueous, ethanolic and methanolic) of *T. procumbens*.

**Keywords:** Agar disc diffusion, biogenic nanoparticles, biomass, bio-mineralization, food poisoning method, FTIR, nanoscale, SEM, XRD

**Introduction**

Nanotechnology is a growing area that takes along with it numerous prospects and possibilities, resulting in the advancement in disease treatment as well as in pharmaceutical and medical field. At a nanoscale, the physical, chemical and biological properties of materials differ fundamentally from their corresponding bulk complement because of the size-dependent quantum effect. These materials or devices are used for the beneficial purposes, while retaining their properties and functionality. Further, the engineered nanomaterials have even enhanced properties as compared to the natural nanomaterials. Nanoparticles containing herbicides, chemicals or genes serve as ‘magic bullets’ capable of targeting particular plant/animal parts to release their content. Surface functionalized, novel metal nanoparticles represent smart and promising candidates in the drug delivery applications because of their unique dimensions, remarkable functionalities on the surface and controlled drug release. Another essential aspect, while working with nanoparticles in their bio-applicability, is safety and biocompatibility. Nanoparticle fabrication can be achieved either by chemical or biological systems. Among the different fabrication methods, green synthesis of metal nanoparticles (NPs) is preferred due to its cost-effectiveness and environmental compatibility. There are many different reports on the biogenesis of silver NPs using plant extracts or their fractions. Some reducing ingredients, such as, polyphenolic compounds, have been known to reduce metal ions into their corresponding NP forms.

*Tridax procumbens* L. (Family: Asteracae), commonly known as ‘Ghamra’ or ‘coat buttons’ in English, is native to tropical America. Later it also naturalized in tropical Africa, Asia and Australia. In India, it is found as a wild herb. It can be easily seen along road sides, waste grounds, dikes, rail tracks, riverbanks, meadows and dunes. The reported phytoconstituents in this plant are alkaloids, carotenoids, flavonoids (catechins and flavones) and

---

*Author for correspondence
Tel: +91-141-3080601-65; Fax: +91-141-2752418
E-mail: cpm_malik@yahoo.com
tannins. Plant is richly endowed with carotenoids and saponins. The proximate profile shows that the plant is rich in sodium, potassium and calcium. This plant is of great importance as it possesses important pharmacological properties like hepatoprotective, immunomodulator, wound healing, anti-diabetic and antimicrobial\textsuperscript{9}. Callus induction in \textit{T. procumbens} was reported from nodes and leaves of the plant. Successful callus induction was obtained using 0.5 mg L\textsuperscript{-1} and 0.2 mg L\textsuperscript{-1} of 2,4-D from nodes and leaves, respectively\textsuperscript{10}.

The intent of the present study was to synthesize and characterize biogenic nanoparticles from the stem and leaf callus of \textit{T. procumbens} and to test them for antimicrobial activity using \textit{Escherichia coli}, \textit{Vibrio cholerae}, \textit{Aspergillus niger} and \textit{A. flavus}. Further, the activities of these synthesized biogenic nanoparticles were also compared with various plant extracts (aqueous, ethanolic and methanolic) of \textit{T. procumbens}.

Materials and Methods

Callus Induction

For callus production, explants (leaves and stem) were collected from plants of \textit{T. procumbens} L. during January-February, 2011. The collected explants were surface sterilized with diluted solution of lavoline, bavistain (0.1% w/v) and mercuric chloride (0.1%), followed by washing with distilled water after each treatment. The explants were then transferred onto MS medium supplemented with vitamins and plant growth regulators (PGR) along with sucrose as a carbon source. The PGRs utilized for inducing of callus from stem and leaves were 2,4-D (0.5 & 0.2 mg L\textsuperscript{-1}, respectively). Callus formation started within 3-4 wk of inoculation. Through regular sub-culturing sufficiently large amount of callus was obtained within 9-10 wk of inoculation.

Preparation of Silver Nitrate Solution and Reaction Mixture

Aqueous silver nitrate solutions (5 mM & 10 mM) were prepared using silver nitrate powder and triple deionized water in a fixed ratio. For the preparation of leaf callus extract, resultant callus was washed with autoclaved water thrice and air dried. The air dried callus (30 g) was crushed in triple deionized water with the help of mortar and pestle, and the final volume was made to 100 mL. The extract was then boiled for 15 min on a hot plate and filtered with the help of Whatman’s Filter paper no. 1. The filtrate was used for further experimentation. The same process was followed to prepare stem callus extract.

The reaction mixture was prepared by taking 10 mL of the prepared filtrates obtained from the plant callus individually (stem & leaf) and 90 mL of above prepared silver nitrate solution (5 & 10 mM). The reaction mixture was then incubated at room temperature for up to 5 h. Observations were made after each hour of incubation to observe changes in colour, ranging from light greenish to dark brownish. After each hour, a small amount of the reaction mixture was centrifuged at 18,000 rpm for 25 min and the pellet was stored at 4°C.

Ultraviolet-Visible (UV-Vis) Spectra Analysis

Synthesized bionanoparticles were characterized by UV-Vis spectroscopy, which is one of the most widely used techniques for structural characterization of silver nanoparticles\textsuperscript{11}. This analysis helps to monitor the bioreduction of pure Ag\textsuperscript{+} ions occurring in the reaction mixture during 1-5 h of incubation. The pellet obtained after centrifugation of reaction mixture was used for the UV-Vis analysis after diluting it in 2 mL of triple deionized water. For the analysis, Genesys 10 UV spectrophotometer was used to screen the reducing power of the Ag\textsuperscript{+} synthesized in the reaction mixture. The final absorbance was recorded from 300 to 600 nm to obtain the characteristic peaks.

Scanning Electron Microscope (SEM) Analysis

SEM analysis of nanoparticles was carried out with EVO-50 INCA Penta FETx 3 machine to characterize mean particle size as well as their morphology. The lyophilized sample of nanoparticles was ultrasonicated with distilled water. A small drop of this sample was placed on carbon stubs and extra solution was removed from the stubs with blotting paper. The prepared stubs were air-dried and subjected to analysis. The machine was operated at a vacuum of the order 10-5 torr and the accelerating voltage of the microscope was kept in the range 10-20 kV.

X-Ray Differection (XRD) Analysis

The size of the bionanoparticles was confirmed by XRD analysis. A thin layer of the sample was prepared on a glass slide and the preparation was subjected to analysis with an ISO-DEBYEFLEX 2002 XRD machine operating at 40 kV voltage and 30 mA current with Cu K\textalpha radiation in 0-2 0 configurations. The crystallite domain size was calculated from the
width of the XRD peaks, assuming that they are free from non-uniform strains, using the Debye-Scherrer formula:

\[ D = \frac{0.94 \lambda}{\beta \cos \theta} \]

Where \( D \) is the average crystallite domain size perpendicular to the reflecting planes, \( \lambda \) is the X-ray wavelength, \( \beta \) is the full width at half maximum, and \( \theta \) is the diffraction angle.

Fourier Transform Infra-Red (FTIR) Spectra Analysis

FTIR analysis was performed by formation of KBr pellet. About 2 mg of the nanoparticle sample was taken in mortar and pestle along with 10 mg of the KBr and mixed. This mixture was then subjected to dye and a hydrolic pressure of about 1.5 bar was applied for few seconds and released. The formed pellet was carefully taken on the sample holder and subjected to FTIR analysis. The analysis was carried out using BRUKER-VERTEX-70 machine and the software used for the analysis of the sample was OPUS.

Antimicrobial Assay

Antibacterial Assay

Agar disk diffusion method was used for antibacterial assay. About 100 \( \mu \)L of freshly prepared culture inoculum of \( E. \) coli in liquid (devoid of agar) Luria-Bertani medium was spread on Petri plates containing solid (with agar) Luria-Bertani medium. A sterile paper disk containing nanoparticles along with 3 (tetracycline, penicillin & streptomycin) control disks were placed on the culture plates. Then plates were incubated at 37°C and observations were made after 48 h of incubation. However, for \( V. \) cholerae, thiosulfate citrate bile salts sucrose agar (TCBS) medium was used. About 100 \( \mu \)L of fresh inoculum was prepared using alkaline peptone water (APW), which is highly specific for Vibrio species and helps to discriminate between Vibrio and non-Vibrio species. The rest of the procedure was same as described above.

In case of food poisoning method, bionanoparticles were added to eosin methylene blue (EMB) medium at the time of pouring of medium into the Petri plates. Solidified plates were then streaked with \( E. \) coli and cultured as described above. Similarly, TCBS medium was employed in case of \( V. \) cholerae. The culture plates having no nanoparticles were used as control. The observations were made after 48 h of culture.

Antifungal Assay

The antifungal assay of nanoparticles was performed against \( A. \) niger and \( A. \) flavus using food poisoning method. Petri plates containing 15 mL of Potato Dextrose Agar (PDA) medium was supplemented with bionanoparticles. Freshly grown mycelium of \( A. \) flavus and \( A. \) niger were point-inoculated into the plates separately and incubated at 28°C for 72 h. The PDA plates devoid of nanoparticles served as control. After 72 h of culture, the diameter of fungal colony was recorded. The inhibition (\%) of fungal growth was calculated by the following formula:

\[ \% \text{ inhibition} = \frac{C - E}{C} \times 100 \]

Where \( C \) is the diameter of fungal mycelium on control plate and \( E \) is the diameter of fungal mycelium on plates containing bionanoparticles.

Comparison with Plant Extracts

The activity of synthesized bionanoparticles was compared against the various plant extracts. For that aqueous, ethanolic and methanolic plant extracts were prepared in varying ratios (30, 40 & 50 ppm). The antimicrobial behaviour of the extract as well as the synthesized bionanoparticles were analyzed by means of disk diffusion method using \( E. \) coli, \( V. \) cholerae, \( A. \) niger and \( A. \) flavus as test organisms.

Results and Discussion

The reduction of silver ions into silver particles following exposure to the callus extracts of \( T. \) procumbens could be deciphered by a change in colour of the reaction mixture, while incubating them at room temperature. Apparently, the metabolites in the callus extracts effectively acted as e' donors and reduced Ag\(^+\) ions to Ag and the formation of nanoparticles was indicated by the brown colour of the aqueous solution, following the excitation of surface plasmon vibrations\(^{12} \) (Figs 1 & 2). The results thus show that \( T. \) procumbens, a medicinally important plant, can be a potential source for synthesizing bionanoparticles. The UV-Vis spectrograph of the colloidal solution of bionanoparticles was recorded as it is a very useful technique for the analysis of nanoparticles\(^{13} \). A slight variation was observed in the spectrograph of nanoparticles from both the sources. The spectral analysis showed that the formation of nanoparticles was initiated during the 2-3 h of incubation, which
was inferred by the absorption peak obtained at 410-430 nm in both the samples of nanoparticles derived from stem and leaf callus extracts (Fig. 3a & b). Further, it also indicated that the synthesized nanoparticles were polydispersed.

SEM analysis of the synthesized bionanoparticles was performed and images received are presented as Fig. 4a & b. No variation was observed between the images received for nanoparticles obtained from both the sources. While considering the results obtained by the above two techniques (SEM & UV-Vis), the average mean size of the nanoparticles was found to be 25 nm (using stem callus extract) and 23 nm (using leaf callus extract). This average size was further confirmed by using the XRD analysis. The XRD pattern showed 3 intense peaks in the whole spectrum of 2θ value, ranging from 0 to 80 (Fig. 5a & b). Like UV-Vis spectra, here also no difference was found between the patterns of nanoparticles from both the sources. The XRD pattern of biogenic nanoparticle clearly illustrates that the synthesized nanoparticles are crystalline in nature.

Our findings substantiate the results obtained from studies with Capsicum annuum\(^{14}\) and Aloe vera extracts\(^{15}\). The reduction of silver ions during the incubation could be easily examined using UV-Vis spectroscopy. Further characterization of synthesized biogenic nanoparticles was ascertained by TEM, SEM and XRD. In our studies, we have used the latter two to characterize the synthesized biogenic nanoparticles. A review of the literature indicates that the size of nanoparticles varied with the species used. For example, it was 8 nm in case of Nicotiana\(^{16}\), 26 nm in Coriandrum\(^{17}\), and from 5 to 30 nm in C. quadrangularis. In the present study, we

Fig. 1 (a & b)—Callus obtained from stem (a) and leaf (b) of T. procumbens.

Fig. 2 (a-d)—Initial reaction mixture from callus extracts of stem (a) and leaf (c), and after 5 h of incubation [(b) and (d), respectively].

Fig. 3 (a & b)—UV-Vis spectra analysis of bionanoparticles obtained from callus extracts of stem (a) and leaf (b).
encountered sizes of 23 nm from leaf and 25 nm from stem callus extracts of *T. procumbens*.

Once the nanoparticles are produced they have the tendency to agglomerate, which largely depends on the chemistry as well as electromagnetic properties. To overcome this agglomeration process, the synthesized nanoparticles are coated with nonmagnetic substances, such as, PVC and thiourea, to maintain their homogeneity. In the present study, PEG acted as stabilizing agent.

FTIR analysis of the synthesized nanoparticles was performed and results are shown in Fig. 6a & b. The FTIR spectra showed absorption bands in regions ranging from 3400 to 850 cm\(^{-1}\) (Table 1). The absorbance bands help in identifying the resultant groups responsible for the formation of the nanoparticles. Carbonyl groups from the amino acid residues and peptides of proteins showed strong affinity for binding with metals, suggesting that the protein could act as an encapsulating agent and, hence, can also protect nanoparticles from agglomeration.

The effectiveness of biogenic nanoparticles (BNPs) as antimicrobial agent against the bacteria, viz., *E. coli* and *V. cholera*, and fungi, viz., *A. niger* and *A. flavus*, was studied. The toxicity was assessed through food poisoning and disk diffusion method using different concentrations (20, 30, 40, 50, and 60 ppm) of BNPs. Among these, 30 and 40 ppm were found to be minimum inhibitory concentrations (MIC) for *E. coli* and *V. cholerae*, respectively (actual data not shown). In disk diffusion studies, BNPs showed antimicrobial
activity against both the bacteria but their efficacy was below par to the antibiotics studied (tetracycline, penicillin & streptomycin). On the other hand, BNPs completely checked the growth of both *E. coli* and *V. cholera* in food poisoning studies. In studies against fungi, BNPs also showed considerable antimicrobial activity against *A. niger* and *A. flavus* (actual data not shown).

It is inferred through various studies that the antibacterial effect of silver nanoparticles is associated with the peptidoglycan layer\(^{18}\), although the precise mechanism of the inhibitory effects on microorganisms is still not vivid. Nanoparticles also affect the Gram-negative bacteria, forming pits in the cell wall and causing the accumulation of Ag nanoparticles in the bacterial membrane leading to an alteration in permeability, and finally resulting in cell death. Moreover, Danilczuk *et al*\(^ {19}\) reported that the antimicrobial mechanism of Ag nanoparticles is somewhat associated with the formation of free radicals. In addition, free uptake of free silver ions may cause the disruption of ATP production and DNA replication\(^ {20}\).

The antimicrobial activities of aqueous, ethanolic and methanolic plant extracts against *E. coli* and *V. cholerae* were also compared with the BNPs synthesized from the callus extracts of *T. procumbens*. Although all the extracts showed some inhibitory activities but they were far inferior to the activities shown by BNPs and silver nitrate (actual data not shown). This clearly shows that *T. procumbens* plants also contains certain constituents those possess antimicrobial activity.

In conclusion, the present investigation demonstrates that extracts obtained from the callus of stem and leaf of *T. procumbens* plants were capable of producing silver nanoparticles from an aqueous solution of AgNO\(_3\). Moreover, these nanoparticles showed considerable antimicrobial activity to bacteria and fungi. Thus, they show great potential for applications in biomedicine, nanomedicines, nanoelectronics, and nanooptical devices.

**Acknowledgement**

Authors would like to thank Rajasthan University, Jaipur and Indian Institute of Technology, Kanpur for providing help and necessary facilities for lyophilization and SEM, XRD and FTIR analyses during the present study.

**References**


