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ORIGINAL RESEARCH PAPER

Antimicrobial and cytotoxicity effect of silver nanoparticle synthesized by *Croton bonplandianum* Baill. leaves

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ABSTRACT

Objective(s): For the development of reliable, ecofriendly, less expensive process for the synthesis of silver nanoparticles and to evaluate the bactericidal, and cytotoxicity properties of silver nanoparticles synthesized from root extract of *Croton bonplandianum, Baill*.

Materials and Methods: The synthesis of silver nanoparticles by plant part of Croton bonplandianum was carried out. The formation of nanoparticles was confirmed by Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), XRD and UV-Vis spectrophotometric analysis. The biochemical properties were assayed by antibacterial study, cytotoxicity assay using cancer cell line.

Results: The formation of silver nanoparticles was confirmed by UV-VIS spectroscopic analysis which showed absorbance peak at 425 nm. X-ray diffraction photograph indicated the face centered cubic structure of the synthesized AgNPs. TEM has displayed the different dimensional images of biogenic silver nanoparticles with particle size distribution ranging from 15-40 nm with an average size of 32 nm. Silver particles are spherical in shape, clustered. The EDX analysis was used to identify the elemental composition of synthesized AgNPs. Antibacterial activity of the synthesized AgNPs against three Gram positive and Gram negative bacteria strains like *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* carried out showed significant zones of inhibition. The cytotoxicity study by AgNPS also showed cytotoxicity on ovarian cancer cell line PA-1 and lung epithelial cancer cell line A549.

Conclusion: The present study confirms that the AgNPs have great promise as antibacterial, and anticancer agent.

Keywords: *Antibacterial activity, Croton bonplandianum, , Cytotoxicity, Silver nitrate, Silver nanoparticles*

INTRODUCTION

A particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Nanoparticles are particles have a diameter between 1 and 100 nanometers [1]. Nanoparticles have several applications like antimicrobial [2], antifungal [3], targeted drug delivery [4] etc. in the field of biotechnology. Biosynthesis of nanoparticles is an interdisciplinary technique build up in combination of nanotechnology and biotechnology. This technique received attention due to it economical and ecofriendly nature [5]. Nanoparticles derived from gold, silver and platinum have medical as well as pharmaceutical applications [6]. Silver nanoparticles have many important applications like an antimicrobial agent, home water purifier, medical devices, cosmetics, textiles etc [7]. Silver nanoparticle may synthesized by various method like reduction in solutions [8], thermal decomposition of silver compounds [9], biological reduction method [10]. Biological Reduction method is one step, environmental friendly method of silver nanoparticle synthesis.*Croton bonplandianum* Baill. is a medicinal herb found in tropical areas in India. [11].

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C. bonplandianum is having wide range of therapeutic importance. In folk medicine, this plant is used to treat skin disease, ring worm infection and to cure the swelling of body [12]. Leaves of this plant have also use in treatment of cuts and wounds, venereal sores as well as in treatment of cholera [13]. The genus *Croton* is enriched in secondary metabolites like alkaloids, terpenoids, as well as possesses toxic components, phorbol esters [14,15]. Phytochemical studies have been reported that, *C. bonplandianum* principally contain rutin ($C_{18}H_{36}O_{19}$] as main constituent, crotsearinine, crotasparine and its methyl derivatives aphorbol [16, 17]. In this study we synthesized silver nanoparticle from *C. bonplandianum* aqueous leaf extract using bioreduction method and evaluated their antibacterial activity. We further studied the cytotoxicity of synthesized nanoparticle against A549 and PA1 cancer cell lines.

MATERIALS AND METHODS

The plant of *C. bonplandianum* were collected from Purba Medinipur, West Bengal, India. Silver nitrate ($AgNO_{3)}$ was obtained from Himedia India. Deionized water was used throughout the reactions that obtained from National Chemical Co., Kolkata, India.

Bacterial strains Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive bacteria *Staphylococcus aureus* were procured from Institute of Microbial Technology, Chandigarh, India.

Preparation of leaf extract

C. bonplandianum plants were collected from the different parts of Purba medinipur, West Bengal, India. They were washed thoroughly first with tap water to remove the soils and dirt, then with sterile double distilled water. The leaves were air dried for a week at room temperature and finally the dried leaves were grinded. Leaf powder was weighed (10 g) and mixed with 100 ml of distilled water. The mixture was boiled in water bath for 10 minutes at 90^0C, after that it was filtered through Whatman No. 1 filter paper to obtain the pure root sample extract. The filtrate thus obtained was used as plant extract.

Synthesis of silver nanoparticles

Synthesis of silver nanoparticles was done as per description of Gavhane et al. 2012 [18] with some modifications. 10 ml of the plant extract was mixed with 90 ml of 1mM aqueous silver nitrate solution for reduction of Ag^+ and boiled for one hour at 90^{0C} in a water bath. The plant extract act as capping as well as reducing agent. Color change of the solution is the indication of synthesis of silver nanoparticles.

The Colloidal solution contain nanoparticles, was centrifuged at 2000 X g for 30 minutes. The pellet was washed in distilled water several times to remove residual silver and other impurities. Finally, nanoparticles were dried in air.

Characterization of nanoparticle

UV-vis Spectroscopy

The reduction of Ag+ ions was recorded by measuring the UV–vis spectrum [19]. Diluting a small aliquot of the sample with distilled water and analysis was done using UV–vis spectrophotometer (Eppendorf Bio Spectrometer) in the range of 200–700 nm.

XRD analysis

The formation and quality of compounds were checked by XRD technique [20].

The sample was completely dried at 60^0 C at hot air oven overnight and subjected to XRD for the determination of the formation of Ag-NPs by a X'Pert Pro x-ray diffractometer (PAN analytical BV, The Netherlands) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in a θ-2 θ configuration.

Scanning Electron Microscopy (SEM)

Scanning electron microscopic analysis of synthesized SNPs was done as per [21]. Dropping a very small amount of the aqueous SNPs on the grid, thin films of the AgNPs were prepared on a carbon-coated copper grid. Excess material were removed using blotting paper.

These films were dried under mercury lamp for 5 min. SEM observations were carried out on a ZEISS EVO 40 EP Electron microscope. The synthesized AgNPs were characterized with the help of scanning electron microscopy (model LEO 440i) equipped with X-ray energy dispersive spectrometer (EDAX).

Antibacterial assay

Bacterial strains Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive bacteria *Staphylococcus aureus* were used in this study. Silver ions as well as AgNPs have strong antimicrobial activities [22]. Antibacterial activity of

AgNPs ware assayed by using the agar well diffusion technique [23].

Luria agar plates were prepared and each of these plates 4 no of 5-mm diameter wells were made. Fresh liquid culture of 10^5 cfu/ml was spread uniformly into separate plates in aseptic condition under horizontal laminar air flow chamber.

Aqueous solution of 2.5, 5, 7.5, 10 of nanoparticles were poured in the wells and allowed to diffuse at room temperature for 30 min.

Ciprofloxacin (0.19 µg/ml) was used as standard antibiotic for the microorganism.

Plates were kept in incubation at 37^0C for 24 hours at inverted position. After incubation, clear inhibition zone around the wells were measure by scale. All antimicrobial activity data are the mean of triplicate analyses.

Cytotoxicity study

Cytotoxicity analyses of silver nanoparticles were performed in A549 and PA1 cell line as per [24].

A549 cells are adenocarcinoma human alveolar basal epithelial cells and PA1 was established from cells taken from ascitic fluid.

These two cell lines were maintained in DMEM, supplemented with 2mM L-glutamine, 1% penicillin-streptomycin, and 10% FCS. Cells were grown at 37^0C in a humidified chamber containing 5% CO^2.Exponentially growing cells was harvested from the culture flasks by trypsinization and then resuspended in fresh medium.

The suspended cells of 5000 cells/well was dispensed into a 96-well micro-plate and be incubated for 24hrs. Then various concentrations of AgNPs (1, 2.5, 5, 7.5, 10 microgram/ml) were used. Each experiment was conducted in triplicate.

The cell viability in the microplate was determined using the MTT (3- (4,5- dimethylthiazol -2- yle) 2,5-diphenyl-tetraloziumbromide) after incubation [25]. MTT was added to each well 5mg/ml concentration.

After incubation for 4 hrs, the cells from each well were solubilized with 100 µl DMSO for determination of optical density at 570 nm.

Statistical analysis

The cell viability study was done in triplicate. The results were presented as mean $\pm$ standard deviation. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of $p<0.05$.

RESULTS

UV–visible spectroscopy of silver nanoparticles

In order to monitor the formation and stability of silver nanoparticles, the absorption spectra of the synthesized silver nanoparticles were recorded against distilled water. (Fig. 2b) shows the UV–visible spectra of silver nanoparticle formation using constant $AgNO_3$ concentration (1mM). The color of the solutions changed from pale yellow to yellowish brown to deep brown depending on the extract concentration indicating silver nanoparticle formation as the color change observed is due to excitation of surface Plasmon vibration in the silver nanoparticles (Fig 2a). It can be seen that the surface Plasmon resonance (SPR) of AgNPs is 425 nm.

X-ray diffraction (XRD) analysis

The crystalline nature of Ag NPs was confirmed by the XRD analysis in Fig 3. The diffraction peaks at 39.12°, 46.44°, 64.84° and 76.87° correspond to the (111), (200), (220) and (311) facets of the fcc crystal structure, respectively. The peak corresponding to the (111) and (200) plane are more intense than the other planes, suggesting that the (111) and (200) plane are the predominant orientation.

Scanning Electron Microscopy and EDX

The silver nanoparticle SEM and XRD were performed of dried samples. SEM micrographs show aggregates of AgNPs. These agglomerated AgNPs forms a spherical shape and the particles are in the range of 15-40 nm in size and the average size is 32 nm (Fig. 4a).

Fig. 4b shows the surface of silver nanoparticles and the elements present in the extract by EDX.

TEM analysis

The surface morphology, size and shape of phytoreduced silver nano particles were characterized and shown in the TEM microphotograph (Fig 5a). It is evident from the photograph that the AgNPs are spherical in shape and polydispersed.

The measured size ranges from 15-40 nm and the average size is 32 nm. Occasional agglomeration has also been observed. Fig. 5b shows the Selected Area Electron Diffraction (SAED) pattern of the silver nanoparticles represented face centered cubic crystalline structure of the silver in the different diffracting planes.

Fig. 1. *Croton bonplandianum* Baill

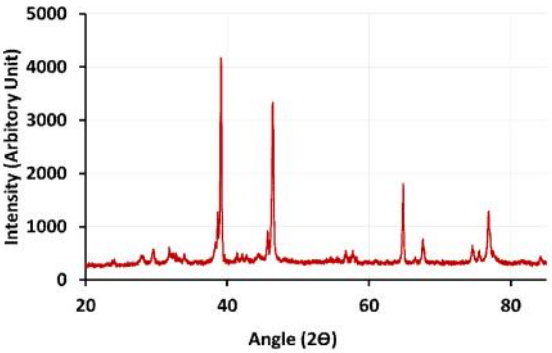

Fig. 3. X-ray diffraction pattern of silver nanoparticles using *C. bonplandianum*

Fig. 2a. Synthesis of Silver nanoparticle: Change in color during synthesis of silver nanoparticle from plant extract

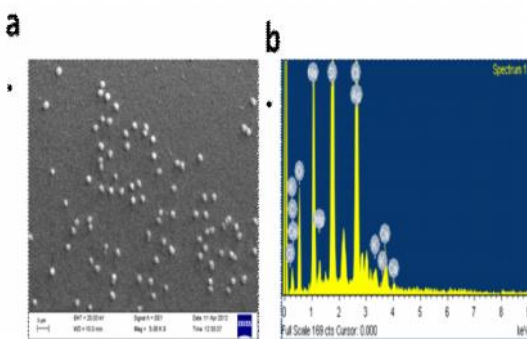

Fig. 4. Scanning Electron Microscopy of silver nanoparticle: (a) Silver nanoparticles are spherical in shape and have an average diameter around 32 nm. (b) EDX profile of nanoparticles synthesized by *C. bonplandianum*

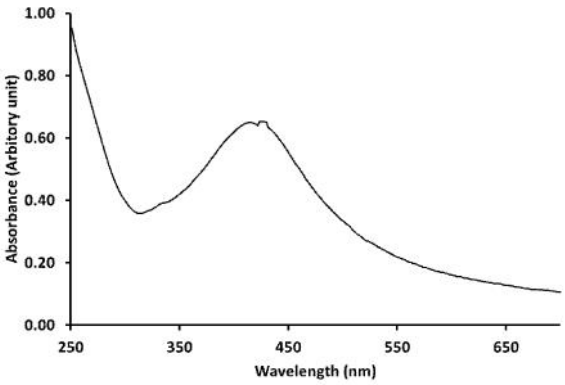

Fig. 2b. UV- Vis spectroscopy of silver nanoparticle:UV–vis spectroscopy of small aliquot of the sample with distilled water in the range of 200–700 nm give a peak at 425 nm

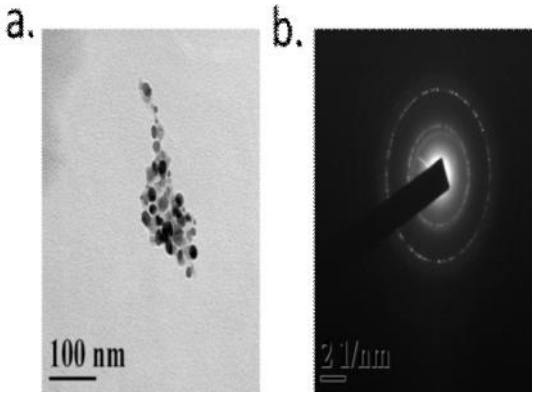

Fig. 5. TEM images of AgNPs by *C. bonplandianum* extract showing (a) spherical shaped
Particles with an average size of 32 nm; (b). SAED pattern of major silver nanoparticles formed by the reduction of Ag+ ions

Antimicrobial assay

The minimum inhibition concentrations of ciprofloxacin for gram positive *S. aureus*is 150 g/ml; for gram negative *E.coli* is 100 g/ml; and for *P. aeruginosa* is 200 g/ml (Fig 6). The results revealed that AgNPs synthesized from C. bonplandianum demonstrated effective antibacterial activity in Gram negative than in Gram positive bacteria. It can be suggested that Gram-negative strains of bacteria *E. coli* and *P. aeruginosa* with thin cell wall is more susceptible to cell wall damage compared to Gram-positive strain bacteria *S. aureus* with a thick cell wall.

MTT assay

In vitro cells assays, PA-1, and A549 cells were exposed the different concentrations of AgNPs of *C. bonplandianum*. The silver nanoparticles produced from crude extract of *C. bonplandianum* showed cytotoxicity against A549 cells lung cancer cell line in dose dependent manner. The results of MTT assays show that AgNPs of *C. bonplandianum* exhibit a significant cytotoxicity in case of A549 cells compared to PA-1 cell lines. Almost 55% A549 cells senesce at 7.5 ?g/ml of AgNPs whereas at that particular concentration of AgNPs, only 30% PA-1 cells senesce (Fig 7).

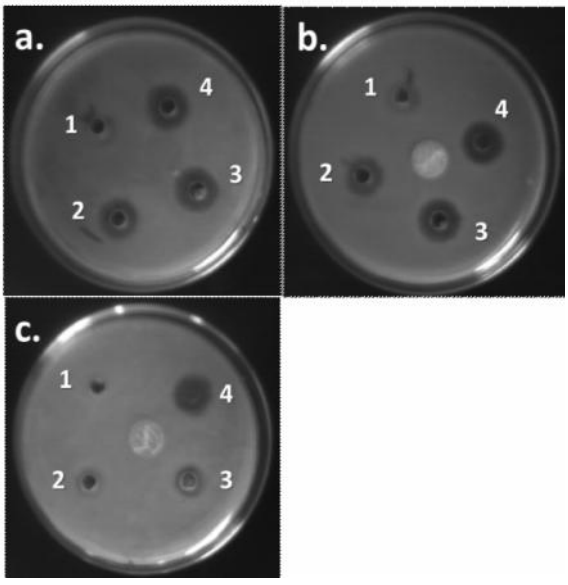

Fig. 6. Antibacteria activity of different concentrations (1:2.5, 2:5,3: 7.5, and 4:10 ml) of *C. bonplandianum* mediated AgNPs a. Petridish with lawn *of P. aeruginosa*, b. petridish with lawn of *E. coli*, c, petridish with lawn of *S. aureus*

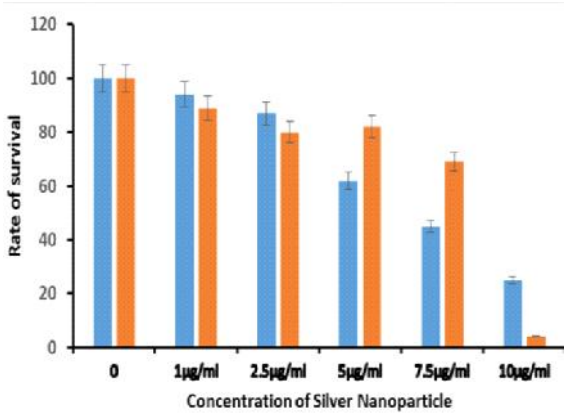

Fig. 7. MTT assay of A529 and PA1. The blue bars are indicate A549 cell line, Maroon bars for PA1 cell line

DISCUSSION

Many research articles reported the synthesis of silver nanoparticles using plant extracts. Plant extracts from different parts of the following plants were used to synthesize silver nanoparticles - *Jatropha curcas* seeds [26]; *Ocimum sanctum* stems and roots [27]; *Nicotiana tobaccum* leaf [28]; *Ocimum sanctum* (Tulsi) leaf [29]; *Ocimum tenuiflorum, Solanum trilobatum, Syzygium cumini, Centella asiatica*, and *Citrus sinensis* leaves [30]; *Trianthema decandra* roots [31]; *Helianthus annuus, Basella alba, Oryza sativa, Saccharum officinarum, Sorghum bicolor*, and *Zea mays* [32]; banana peel [33]; *Chenopodium album* leaf [34]; *Ficus benghalensis* leaf [35]; and mulberry leaves [36]. *Croton bonplandianum* is an exotic weed which is found in the wastelands. The leaves of this plant have medicinal values. They are used as antiseptic agent for the treatment of any cuts and wounds [37-39]. Because of its wide usage and availability in the rural area, this study was adopted to investigate the antimicrobial and anticancer efficacy of the nanoparticles synthesized from the leaves of this plant. The synthesized silver nanoparticles from C. bonplandianum leaf extracts in the colloidal solution were monitored by UV–vis spectrophotometer analysis. Fig 2b shows that the absorption spectra of silver nanoparticles. The absorbance peak at 425 nm correspond to the surface plasmon resonance of silver nanoparticles. X-ray diffraction results clearly indicate that the silver nanoparticles formed by the reduction of Ag+ ions by the C. bonplandianum leaf extract are crystalline in nature. A peak was also observed at 2è

equal 22 suggesting that the crystallization of bio-organic phase occurs on the surface of the silver nanoparticles [40]. Therefore, from the XRD pattern it is clear that AgNPs formed using the c. bonplandianum leaf extract were crystalline in nature. Scanning electron microscopy has provided insight into the morphology and size details of nanoparticles.

The SEM micrograph shows that synthesized silver nanoparticles were well dispersed without any aggregation. They possesses spherical shape. The SAED spectra from the AgNPs were recorded. The SAED profile shows a strong silver signal with signals of oxygen, Na, Si, Cl which may have originated from the biomolecules bound to the surface of the silver nanoparticles. Another reason for the presence of these unwanted signals may be due to the same present in the grids.

It has also been reported that the thin layer of capping organic material from the plant extract surrounds the nanoparticles which makes them stable in solution for month. The particle sizes were in the range of 15-40 nm with an average size of 32 nm which were confirmed by SEM, TEM, and XRD.

The minimum inhibitory concentrations of synthesized nanoparticles were found to be 50, 45, 75 g/ml in case of *E.coli, P. aeruginosa*, and *S. aureus* respectively. The present data agree well with the work of other researchers [41]. It can be suggested that Gram-negative strains of bacteria *E. coli* and *P. aeruginosa* with thin cell wall is more susceptible to cell wall damage compared to Gram-positive strain bacteria *S. aureus* with a thick cell wall. The positive charge on the silver ion is the cause for antimicrobial activity. It can attract negatively charged cell membrane of bacteria through electrostatic interaction [42-43].

It is also confirmed that AgNPs have significant antimicrobial activity against gam negative bacteria compared to gram positive bacteria hence have a great potential in the preparation of biomedical application.

The in vitro cytotoxicity effects of silver nanoparticles were screened against two different human cell lines-A549, and PA-1 by means of MTT assay. It has been shown that AgNPs reduces ATP content of the cell by damaging mitochondria, thereby increasing production of reactive oxygen species (ROS) in a dose dependent manner [44]. Hence we decided to study the toxicity of silver nanoparticles at different concentrations (1, 2.5, 5, 7.5, 10 g/ml). Similar result was obtained using the silver nanoparticles synthesized using *Scoparia dulcis* [45]. The toxic behavior of green synthesized AgNPs against A549 cells compared to PA-1 cells also showed that they have a potential for acting as anticancer drug in the future.

CONCLUSION

The present study confirms that the silver nanoparticles produced by the leave extract of *Croton bonplandianum* have great promise as antibacterial and anticancer agent.

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