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Antimicrobial activity of biogenic silver nanoparticles synthesized using Tridax procumbens L.

Ondari Nyakundi Erick and M. Nalini Padmanabhan*

Department of Biotechnology, Karpagam University, Coimbatore 641 021, India
**Corresponding author*

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A B S T R A C T

Resistance emergency has necessitated continuous search for drugs with least toxicity, effective, affordable and eco-friendly which possess new mode of action to cater the rising emergency. In this study, *T. procumbens* was used for biogenic synthesis of silver nanoparticles and investigated for antibacterial and antifungal activity. Characterization was done by UV-Vis spectrum, SEM, XRD, EDAX and FTIR. The UV-vis spectrum λ_{max} was observed at 452 nm. EDAX AgNPs revealed total yield of 96.7% with 100 % purity, whilst SEM and XRD revealed spherical and elongated shape with an average grain size of 3.973 nm. FTIR examination provided evidence for presence of primary amides, aromatic ketones and aliphatic amines groups that could be responsible for synthesis and stability of the synthesized silver nanoparticles. Bio potential analysis of the synthesized biogenic silver nanoparticles showed antimicrobial activity against the twelve human and fish pathogens tested with ≥ 10 mm zone of inhibition and among the eight fungal strains involved in this study, *Penicillium restrictum* MTCC 3391 and *Trichoderma virens* MTCC 794 recorded inhibition. The findings recommend the use of *T. procumbens*, a weed plant for biogenic nanoparticle production and can be applied as antimicrobial agent. Isolation and identification of the molecules acting as stabilizers and reducing agents is the scope of further research.

Introduction

The production of biogenic metallic nanoparticles (NP) with least harmful effects to both humans and environment is scaling up recently. Biogenic process involves the use of biological systems like plants, bacteria and fungi in mediating synthesis of NPs using gold, silver and other precursors.

Silver emerges the budding choice not only owing to its wide applications to both flora and fauna but also, in medicine (1). Most importantly, green production is cheaper, environmental friendly and the bio-silver produced is non-toxic (2-4). Overall, bimetallic nanomaterial have wide applications such as catalysts (5), staining

pigments, antimicrobials (6), larvicidal and pupicidal (7), anti-malarial (8), coating on solar cells, photonics (9), sensors (10), in drug delivery, gene therapy, DNA analysis and cancer treatment (11). Though biogenic production process tops the list, it faces stiff competition against physical and chemical production technologies which is attributed to controlled size, shape, robustness and enormous production. However, the two methods remain limited due to production of hazardous compounds being released into the environment, out-dated, complicated, energy consumption and on the grounds that they have limited applications in medical-clinical field (11). Hence, green synthesis remains the only secure choice as plants possess varied secondary metabolites responsible for reduction and stabilization of NPs. In order to achieve maximum production of these bimetals with desired properties for various applications, extensive research should address queries such as production of stable compounds, optimize production parameters, techniques to isolate and identify the compounds responsive for reduction of bioactive molecules to aid mass production and genetic manipulation and will also shed light on molecular and cellular level of these compounds (12) present in respective plant species.

T. procumbens (Fig. 1 inset A) a native to tropical America is recognized for its countless pharmaceutical applications. This weed plant is used by ethno-medical practitioners as a remedy against various diseases and is well documented (13, 14). The plant is effective in treatment of liver disorders, hypertension, bronchial catarrh, malaria, diarrhoea, headache, wound healing, obesity, jaundice, conjunctivitis and haemorrhage from skin injuries. The floral part has been reported for pharmaceutical properties such as antiseptic, insecticidal, parasiticidal and anti- hair falling thereby promoting its growth. Pharmacological activities in literature include immune-modulatory, anti-diabetic, anti-hepatotoxic, anti-oxidant, anti-inflammatory, analgesic, anti-depressant respiration and anti-cancer (15). To date, green synthesis and its various biological pharmaceutical applications in literature are immense (16). Therefore, the present work focuses on green synthesis of silver nanoparticles using leaf extract of *T. procumbens*, characterization and further tested for antimicrobial and antifungal activity against Gram positive and negative bacteria from human and fish pathogens.

Materials and methodos

Extract preparation from *Tridax procumbens*

Crude extract

Fresh and healthy leaves of *T. procumbens* (Fig. 1 inset A) collected from our University campus were washed thoroughly in running tap water, rinsed thrice with distilled water and dried with paper towel. Ten grams of leaves were crushed well in sterile mortar and pestle, centrifuged (11, 000 × *g*, 4ºC, 20 min) and the supernatant (=crude extract) was used for phyto-chemical screening according to Trease and Evans 1989 (17).

Aqueous filtrate

Ten grams of the leaves were cut into small pieces, suspended in 100 ml of sterile deionized water and held in a water bath (60°C, 1 h). This aqueous extract was filtered using Whatmann filter paper (No. 1), centrifuged (11, 000 × *g*, 4ºC, 20 min), the supernatant (=aqueous filtrate) was used to synthesize AgNPs.

Biogenic synthesis of AgNPs

The aqueous filtrate (10 ml) obtained as above was added to 90 ml of $AgNO_3$ solution to give a final concentration of 1 mM, kept under room temperature for reaction time and change of colour was monitored.

Characterization

The bio-reduction of silver ions in *T. procumbens* was monitored by absorbance measurement using UV-Vis spectrophotometer (Shimadzu UV-2450, Japan) to analyse the surface plasmon vibration following colour change at wavelength of 200-800nm with 1nm resolution. Subsequently, the synthesized silver nanoparticles were washed thrice by centrifugation (11, 000 × *g*, 4ºC, 20 min) using deionized water. The pellet was subjected to Shimadzu XRD-6000/6100 with the following parameters: 30kv, 30mA with Cu Kα1 radians at 2 theta (θ). Debye Scherer's equation (D- 0.94λ/βcosθ) was calibrated to determine the average size of the synthesized AgNPs whilst SEM connected with Thermo EDAX was performed to analyse the elemental functional groups following coating on carbon film. For SEM (JEOL-MODEL 6390) samples were prepared by coating on copper grid for 5 minutes under mercury lamp. FTIR (Shimadzu, Japan) was deployed to elucidate the possible functional groups constituted in NP synthesis.

Antibacterial activity

Antibacterial analysis was done by agar disk method according to Bauer *et al.* 1966 (18) with slight modifications. Both human and fish pathogens which served as experimental microorganisms in this study constituted Gram positive (*Micrococcus lysodeikticus* ATCC 4698, *Streptococcus epidermis* MTCC 3382, *Pseudomonas aeruginosa* MTCC1688, *Klebsiella pneumonia* ATCC 4352, *Staphylococcus aureus* MTCC 87) and Gram negative (*Salmonella paratyphi* MTCC 735, *Salmonella typhi* MTCC 1245, *Aeromonas hydrophila* MTCC 646, *Vibrio vulnificus* MTCC1145, *Vibrio parahaemolyticus* HQ693275.1, *Proteus mirabilis* MTCC 1429, *Escherichia coli* MTCC 46) bacteria. Nutrient broth agar plates were prepared and 100 µl of overnight pure cultures were plated using sterile L-rod. The biogenically synthesized AgNPs (50 µl) were loaded onto each disk (0.5 cm) and placed in each culture plate. Disks loaded with 10 µl of streptomycin served as control. All the plates were incubated at 37°C for 12-18 h and the diameter of zone of inhibition was measured in millimetres.

Antifungal activity

Antifungal activity was carried out as per Wang *et al.* 2006 (19) with slight modification. Experimental fungi involved in study were: *Beauveria bassiana* MTCC 2028, *Paecilomyces lilacinus* PDBC PL55, *Penicillium chrysogenum* MTCC 5108, *Penicillium restrictum* MTCC 3391, *Trichoderma harzianum* MTCC 3841, *Trichoderma virens* MTCC 794, *Trichoderma viride* MTCC 167 and *Verticillium lecanii* MTCC 3692. Potato Dextrose Agar (PDA) plates were prepared and point inoculated and kept for incubation at 28°C. Mycelia growth of more than 1 cm was considered sufficient for antifungal activity. The biogenically synthesized AgNPs (50 µl), biogenic AgNPs plus aqueous filtrate (of equal volume=50 µl) were loaded onto each disk (0.5 cm) and 10 µl of ketoconazole served as control. Following incubation for 2-3

days at 28°C and zone of inhibition was observed.

Results and Discussion

Phyto-chemical screening and biogenic synthesis

Phyto-chemical screening revealed presence of saponins, tannins, steroids, terpenoids, alkaloids, flavonoids and cardiac glycosides (Table 1) which is consistent with earlier findings (20). The change of colour from pale greenish-yellow to brownish after 2 h was a confirmatory sign for nanoparticle synthesis by aqueous filtrate of *T. procumbens* (Fig. 1 inset B). On the other hand, there was no change in the reaction mixture containing the pellet of aqueous filtrate (Fig. 1 inset C). The UV-Vis spectrum of the silver nanoparticles dispersed in the reaction mixture is shown in (Fig. 1) with an absorbance wavelength of 452 nm. The findings concur with earlier absorbance reports ranging between 430 nm and 460 nm for AgNPs. Earlier findings involving same plant has shown synthesis within 25 mins of sunlight exposure suggesting temperature plays a vital role in scaling up the reaction (21).

Structural and morphological characterization

The UV-Vis spectrum absorbance is due to the characteristic Surface Plasmon Resonance which is dependent on size and shape of the particle. Formation of single Surface Plasmon Resonance band indicates that the particles are spherical in shape which was further confirmed by SEM. Also, the broad peak observed suggests formation of poly-dispersed particles indicating a wide range of the nanoparticle sizes. SEM revealed that majority of the synthesized silver nanoparticles was spherical and an accumulation led to elongated shape (Fig. 2 A). Accumulation of two or more reducing moieties bound on the surface of the preformed nuclei of particles could have contributed to the formation of elongated large spherical nanoparticles (23). EDAX examination (Fig. 2 B) clearly indicated that the crystal nanoparticles produced was 100 % pure with a total yield of 96.7%. XRD diffraction (Fig. 2 C) reflect pattern features appearing at 2 theta=35.50°, 32.00º 39.00°, 46.00° are indexed according to Bragg reflections as (98), (101), (111) and (200) respectively are in line with those reported in face centered cubic (fcc) of silver confirming crystalline nature.

The average grain size according to $D=\cap\lambda/\cos\theta$ where $\cap= 0.94$, $\lambda=1.54\ 10^{-10}$ calibration (24) was found to be 3.793 and is within the reported range <20 nm (19). The FT-IR band intensities in different regions of the spectrum for the control and test samples, before (Fig. 2 D) and after reaction with $AgNO_3$ (Fig. 2 E) were analysed to reveal the compounds responsible for NP synthesis. There was a shift in the following peaks: $3352–3400^{-1}$ attributed to primary amide group, $1587–1558^{-1}$ due to NH_3 vibrations, $1396–1375^{-1}$ corresponding to benzene ring, $1066–1095^{-1}$ corresponding to C-C-C stretch (aromatic ketone) and $794–775^{-1}$ for C-N stretch (aliphatic amines), which implicated that these groups maybe involved in the process of NPs synthesis. As stated earlier, *T. procumbens* is rich in saponins, tannins, steroids, terpenoids, alkaloids, flavonoids and cardiac glycosides (19) and the functional groups associated with proteinaceous matter may be involved in reducing Ag^+ to Ag^0. Biological components are known to interact with metals via these functional groups and mediate their reduction to NPs (24).

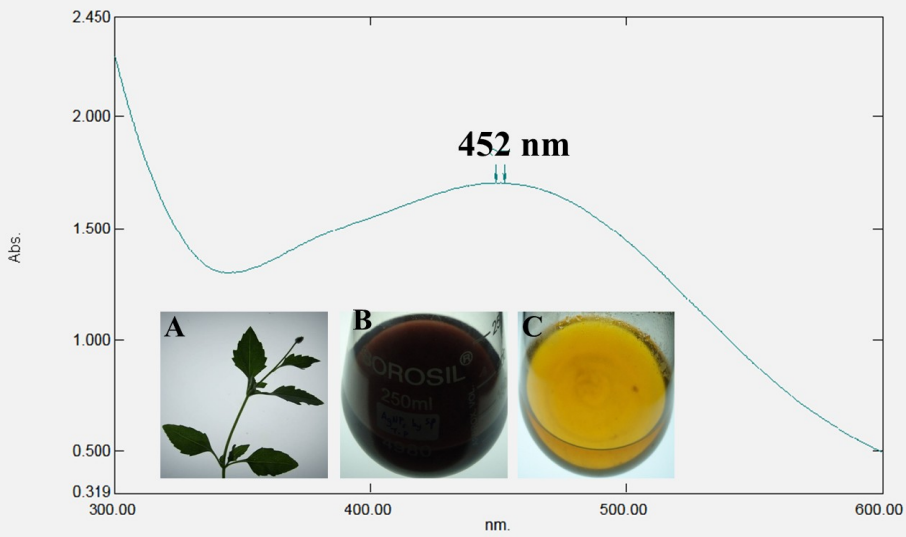

Fig.1 Biogenic synthesis of AgNPs using *Tridax procumbens.* UV-Vis absorbance spectrum **inset**: A. *Tridax procumbens* B. Synthesized biogenic AgNPs using aqueous filtrate (brownish) C. Pellet of aqueous filtrate depicting non-formation of AgNPs (pale yellow).

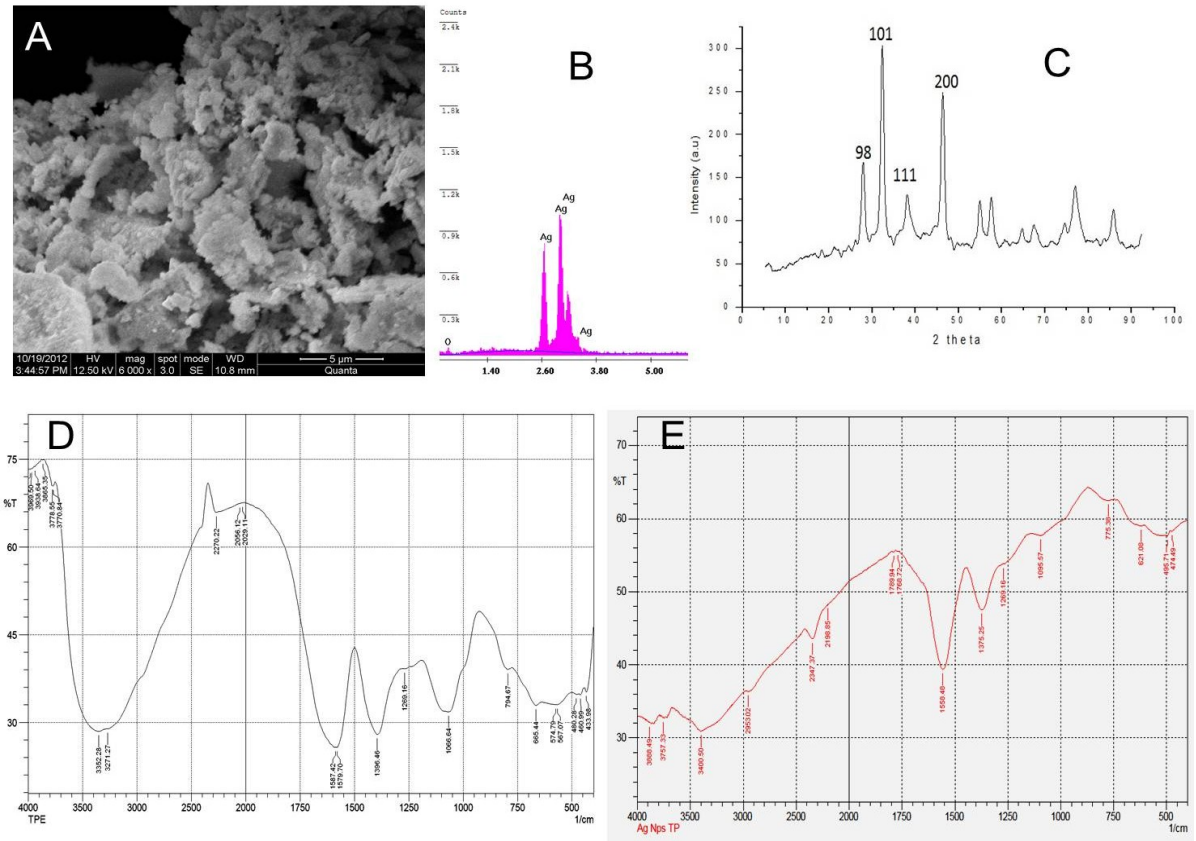

Fig. 2 A. SEM micrograph; B. EDAX spectrum; C. XRD spectrum; D. FT-IR of *T. procumbens* spectrum; E. FT-IR spectrum of biogenic AgNPs

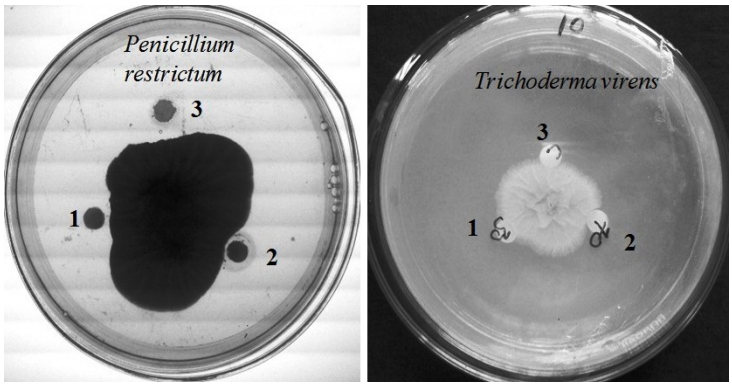

Fig. 3 Antifungal activity where 1. Biogenic AgNPs + aqueous filtrate 2. Biogenic AgNPs 3. Ketoconazole

Table.1 Phyto-chemical screening of *Tridax procumbens* crude extract

Phyto-chemicals	Inference
Saponin	+
Tannins	+
Steroids	+
Terpenoids	+
Alkaloids	+
Anthraquinones	-
Flavonoids	+
Cardiac glycosides	+

+ : Present, - : Absent.

Table.2 Antibacterial activity

Experimental strains	Zone of inhibition (mm)	
	Streptomycin (100 µg.ml^{-1})	AgNPs (5 µg.ml^{-1})
Gram positive		
Micrococcus lysodeikticus ATCC 4698	26	10
Streptococcus epidermis MTCC 3382	31	12
Pseudomonas aeruginosa MTCC1688	25	10
Klebsiella pneumonia ATCC 4352	28	12
Staphylococcus aureus MTCC 87	28	11
Gram negative		
Salmonella paratyphi MTCC 735	25	11
Salmonella typhi MTCC 1254	25	13
Aeromonas hydrophila MTCC 646	35	15
Vibrio vulnificus MTCC 1145	34	12
Vibrio parahaemolyticus HQ693275.1	35	15
Proteus mirabilis MTCC 1429	25	10
Escherichia coli MTCC 46	30	14

Antibacterial and antifungal examination

For many decades, silver has been known for its antimicrobial prospective and its speculated that the AgNPs exert their effects by inhibiting enzymatic respiratory system of microbes, alteration of DNA replication and interaction with S-H bonds of proteins leading to inactivation (25, 26). However, the mechanisms are still obscure, Chamakura *et al.* 2011 (27) have demonstrated how *E. coli* cells absorb AgNPs through cytoplasmic membrane. These AgNPs are also involved in formation of reactive oxygen species thereby inhibiting respiratory enzymes and proteins leading to physiological malfunctioning responsible for mortality of *E. coli* (28).

Therefore, it is speculated that the ingestion of the synthesized silver nanoparticles, lead to death of the tested organisms in the current study. In addition, the surface area exposure of AgNPs to bacteria is dependent on its size which plays a vital role in bacterial mortality. Thus, the average grain size of 3.973 nm attained in the present study, support the bactericidal activity observed against the entire tested human and fish pathogens as well as the fungal strains. Earlier, reports show that nanoparticles of <10 nm were as potential as antimicrobial agents. The synthesized AgNPs exhibited ≥ 10mm zone of inhibition at 50 µg/ml concentration (Table 2) and fungicidal activity observed for *Penicillium restrictum* MTCC 3391 and *Trichoderma virens* MTCC 794 is depicted in Fig. 3. Park *et al.* 2011 (29) have shown that smaller NP have a great ability to induce apoptosis in Mc3T3 cell line than large AgNPs (30) whilst human embryonic stem-cell derived fibroblasts showed higher cytotoxicity compared to L-929 cell lines (31). Thus, the overall size of the nanomaterial produced played major role in medical applications. Hence, further production of AgNPs should focus on size. Combination of standard drugs with AgNPs have justified increased antimicrobial activity, biogenic synthesis of NP will provide helpful insight into development of new antimicrobial agents. The synergy exhibited in combining either plant extracts with silver or commercial drugs is a paramount for the control of harmful microbial population as demonstrated by Durán *et al.* 2007 (32), in which *Fusarium* mediated extracellular production of AgNPs and its incorporation into textile fabrics has shown minimized infection with pathogenic bacteria *Staphylococcus aureus.*

This study reveals the synthesis of pure, small and crystalline AgNPs using *T. procumbens*. The rapid reduction of the AgNPs could be due presence of amines, alkane, proteins, carboxyl and alcohol groups. Antimicrobial activity involving 12 bacterial and 8 fungal strains suggest further development of antimicrobial agents in future for both humans and fish which can be combined with the drugs/feed already in market for higher efficacy. Isolation and identification of individual compounds responsible for synthesis will be of great importance to enhance design for biogenic synthesis of AgNPs with desired characteristic features thus providing an eco-friendly alternative for bio-silver production for medical applications.

Conclusion

The work hypothesized to formulate a more novel use of *Tridax procumbens,* a weed plants' involvement in silver metal reduction and its application against selected bacterial and fungal pathogens.

Significant antimicrobial activity was recorded suggesting application of these synthesized nanoparticles as a potential antimicrobial agent with ≥10 mm zone of inhibition for bacteria as well as fungi *Penicillium restrictum* MTCC 3391 and *Trichoderma virens* MTCC 794. Characterization revealed formation of pure crystalline spherical-shaped AgNPs with an average grain size of 3.973 nm. FTIR analyses revealed primary amides, aromatic ketones and aliphatic amines played role as potential reducers and stabilizing agents.

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Conflict of interest

None declared

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