FTIR Based Phytochemical Profiling And Antibacterial Evaluation Of Green Synthesized Agnps, Cunps And Bimetallic Ag-Cunps Using Aqueous Stem Extract Of Achyranthes Aspera

Boyini Sirisha [1-2*] and Ch. Venkataramana Devi[2]

1. *Telangana Social Welfare Residential Degree College for Women, Mahendrahills, Hyderabad.*
2. *Department of Biochemistry, UCS, Osmania University, Hyderabad.*

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Abstract:

Researchers now-a-days continue to face significant challenges in developing effective antibacterial drugs, as microorganisms increasingly develop resistance to existing antibiotics. The synthesis of nanoparticles from plant extracts represents a significant progress in the field Nanotechnology. Our present study focuses on green synthesis of Metallic nanoparticles (MNPs)- AgNPs, CuNPs and bimetallic Ag-Cu NPs using aqueous stem extract of Achyranthes aspera, chosen for its non-toxic, cost-effectiveness and eco-friendly nature. As a preliminary step, phytochemical analysis of Achyranthes aspera stem extract was conducted to identify the presence of phyto-constituents such as Phenolic compounds, flavonoids, alkaloids, tannins, which plays a critical role in the reducing, capping, and stabilizing the nanoparticles. Fourier Transform Infra-Red (FTIR) spectroscopy analysis was employed to profile these phytochemicals. The antimicrobial activity was evaluated at various concentrations against Gram positive bacteria (Staphylococcus aureus and Streptococcus pneumonia) and Gram-negative bacteria (Pseudomonas aeruginosa and E. coli) using Agar Well Diffusion Assay. Anti-bacterial efficacy was assessed by measuring the diameter of the inhibition zones (ZOI). The microbial activity of the bimetallic Ag-Cu NPs was found efficient against all the tested pathogens, therefore, has a promising utility of green synthesized nanoparticle as an alternative to standard antibiotics, mainly in preventing multi-drug-resistant bacterial strains.

Introduction:

Over the past few decades' nanotechnology has advanced in synthesis of nanoparticles and its applications in science and technology. Nanotechnology is a branch of technology dealing with design, synthesis, characterization, and application of materials within Nano-scale range (1nm -100nm). Nanoscale dimension provides nanoparticles a large surface area to volume ratio and thus very specific properties [1]. Since, last few years chemical methods are used for the production of large quantities of nanoparticles which are complicated, outdated and expensive which produce hazardous toxic wastes that are harmful to environment as well as human health. An alternative method called as 'Bio-based approach' means Nanoparticle synthesis by biological method without addition of any reducing agent and the stabilizer are replaced by molecules produced by living organism i.e. bacteria, fungi, yeast, algae, higher plants etc. These biological sources derived nanoparticles are ecofriendly, more efficient in its biomedical application, easy in production, non-toxic, less time taking and cost-effective nanoparticles [2].

In our study, plant species Achyranthes aspera (common names: chaff-flower, prickly chaff flower, devil's horsewhip, Sanskrit:apāmārga, Telugu: uttareni) belonging to the Amaranthaceae family was used for nanoparticle green synthesis. It can be found all across the tropical regions. With woody stems at the base, Achyranthes aspera is a perennial herb that can grow up to 2 meters tall and occasionally sprawl. It has simple, oval-shaped, opposite, short-stalked leaves that taper to a point at both ends and can have dense or sparse hair. The tiny, greenish-white flowers, which are frequently tinged purple-red, grow into terminal spikes that are initially compact but can grow up to 60 cm in length. This plant has a variety of therapeutic applications used to treat various diseases [3-4].

Taxonomic classification:
Kingdom – Plantae
Subkingdom - Tracheobinota
Super Division - Spermatophyta
Division - Mangoliophyta
Class - Mangoliophsida
Subclass - Caryophyllidae
Order - Caryophyllales
Family - Amaranthaceae
Genus - Achyranthes
Species - Aspera


Achyranthes aspera

The overuse of broad-spectrum antibiotics, unnecessary prescriptions, inadequate administration of antibiotics, and unfinished antibiotic therapy courses have all contributed to the emergence of antibiotic resistance as a major worldwide concern in recent years. The ability of different bacterial strains to withstand the effects of traditional antibiotics has made it necessary to investigate different approaches for managing bacteria that are resistant to these drugs. Studies have shown that antibiotic-infused nanoparticles have many important advantages; in fact, because of their superior antimicrobial qualities, these nanoparticles are increasingly being recognized as nanoantibiotics [5-6]. In this report, the Achyranthes aspera aqueous stem extract was utilized for the preparation of AgNPs, CuNPs, and bimetallic Ag–Cu nanoparticles (NPs) and their antibacterial activity by agar well diffusion assay.

Materials and Method

Collection of the plant

Achyranthes aspera plant was collected from local area of Ghatkesar and authenticated by Department of Botany, Osmania University, Hyderabad. After collection of plant washed thoroughly under running tap water until to the remove of adhering dust particles from the surface of the plant and separated the stems from the plant. Separated plant stems were shade dried and grinded separately into powder for the further experimental analysis [9].

Preparation of Aqueous Stem Extract of Achyranthes aspera

Following the gathering of the plant was thoroughly cleaned under running tap water to eliminate any remaining dust particles from its surface. For the purpose of additional experimental study, the separated plant stems were shade-dried and ground into fine powder. 25g of dry coarsely powdered stem material was submerged in a 250ml of double distilled. The mixture was heated for 20 minutes at 60°C while stirring occasionally. The mixture is cooled to room temperature and filtered using Whatman No.1 filter paper. The filtered extracts of stem were stored at 4°C for further experimental analysis [10].

Phytochemical analysis of phytoconstituents:

The Phytochemical screening of all six extracts was performed by the standard procedures [13].

Test for alkaloids:

a) **Mayer's Test-** Test solution (1 ml) was taken in test tube and few drops of Mayer's reagent (Potassium mercuric iodide solution) were added into it and cream color precipitate was observed.
b) **Dragendroff's Test**- Test solution (1 ml) was taken in test tube and few drops of Dragendroff's reagent (Potassium bismuth iodide solution) were added into it and observed for reddish brown precipitate.

Test for tannins:

a) **Ferric chloride test ($FeCl_3$) Test-** About 0.5 mg of dried powdered samples were boiled in 20 ml water in test tubes and filtered. A few drops of 0.1 % ferric chloride solution was added and observed for brownish green or blue-black coloration.

Test for cardiac glycosides:

a) **Keller-Killiani Test-** Test solution (1 ml) was taken in a test tube and 0.4 ml of glacial acetic acid containing a trace amount of ferric chloride was added to it. Carefully added 0.5 ml of concentrated sulphuric acid by the side of the test tube and observed for blue color to appear in the acetic acid layer.

Test for Steroids:

a) **Liebermann–Burchard test-** Test solution of 1 ml was treated with few drops of acetic anhydride, boiled and cooled, concentrated sulphuric acid was added from the sides of the test tube and observed for a brown ring at the junction of the two layers and green layer in upper layer.

Test for Flavonoids:

a) **Alkaline Reagent test-** About 1 ml test solution was treated with few drops of sodium hydroxide solution and observed for intense yellow coloration which disappeared on the addition of dilute HCl.

Test for Terpenoids:

a) **Salkowski Test-** Test solution (1 ml) was taken in a clean and dried test tube and 2 ml chloroform and few drops of sulphuric acid were added into it. Shaken well and allowed to stand for some time and observed for reddish brown color at interface.

Test for Proteins:

a) **Ninhydrin Test-** Test solutions were boiled with 0.2 % solution of ninhydrin and observed for violet color to appear.

Test for Reducing Sugars:

a) **Fehling's Test-** Test sample of 1 ml was taken into a clean and dried test tube and 0.5 ml of Fehling A and Fehling B solutions were added to it, boiled and observed for brick red coloration.

Test for Saponins:

a) **Froth test-** Test solution (1 ml) was placed in a test tube containing water and shaken well and noted for a stable froth that persists for at least 2 min.

Synthesis of AgNPs, CuNPs and bimetallic Ag-Cu NPs:

The AgNP is synthesized using 10ml of stem extract and added to 90ml of 1mM aqueous $AgNO_3$ solution, followed with heating at 80°C for 3 h with constant stirring. The change in the colour from yellow to brown indicates the formation of Silver Nanoparticle [11]. The synthesis of A. aspera copper nanoparticles, 50 mL (5 mM) copper sulfate solution was mixed with 5 mL of aqueous stem extracts. The pH value 7.0 adjusted for the mixture by the addition of NaOH (1N) solution. Further, the green color mixture was obtained indicates the formation of copper nanoparticle [12]. The equal proportions of AgNP and CuNP were combined to produce the bimetallic Ag-CuNP. All the nanoparticles were stored at 4 °C until further experimental analysis.

Fourier transform infrared (FTIR) spectroscopy analysis of aqueous stem extract of Achyranthes aspera

Fourier transform infrared (FTIR) technique was used for the identification of different functional groups in the extract. The infrared spectroscopy (IR) spectrum was obtained using FTIR using Bruker optics, Germany (model; TENSOR 27). The sample was scanned at a resolution of 4 cm^{-1} at a range of 400-4000cm^{-1}[15].

Antimicrobial activity by Agar well diffusion assay

The bacterial strains Gram positive of Staphylococcus aureus (ATCC 25923),Streptococcus pneumonia (ATCC 33400), and Gram negative of Pseudomonas aeruginosa (ATCC 27853), E. coli (ATCC 25922) used in the study were obtained from ATCC. To conduct the antibacterial activity Nutrient agar plate is prepared and Nutrient broth is prepared for inoculum. The preparation was carried out in the sterile condition and was autoclaved at 121°C for 15 mins before the experimentation. The selected bacterial pathogens were inoculated into nutrient broth and incubated at 37ºC for 24 hours and the suspensions were checked to provide approximately 1-2x10^8 CFU/mL. Media was poured into the sterilized petriplates and let it for solidify. The bacterium inoculum of 100 µL were evenly spread on Muller Hinton Agar plates using a sterile glass spreader. The wells of 6mm diameter were punched in the agar using gel puncture. Four concentrations (25, 50,75 and 100 µl) of aqueous stem extract of A. aspera mediated AgNPs, CuNPs, and Ag-CuNPs each, were tested against different bacterial pathogens. The plates were incubated at 37°C for 18-24 hrs and end of the experiment the diameter of the inhibition zone (mm) was measured around each well [16, 17]. The readings were taken in three different fixed directions and the average values were recorded.

Results and Discussion

This study investigated, the biosynthesis of silver nanoparticle (AgNPs), Copper nanoparticles (CuNPs) and Bimetallic Silver-Copper nanoparticle (Ag-CuNPs) of Achyranthes aspera and evaluated their antimicrobial activity.

Phytochemical Analysis

The phytochemical compounds of the aqueous stem extract of Achyranthes aspera are responsible for the reduction of silver and copper to their respective silver and copper nanoparticles, and these constituents are analyzed qualitatively following the standard method [13]. Our findings reveals that stem extracts of A. aspera contain Phenols, alkaloids, tannins, steroids, flavonoids, terpenoids, and saponin in both significant and trace quantities(Table 1). Achyranthes Aspera is a significant source of novel active biological compounds that exhibit various activities like including anti-microbial, anti-cancer, anti-inflammatory, antiviral, antibacterial, Hepatoprotective, and anticoagulant effects; apart from antibacterial activity [16-20].

Table 1. Qualitative Phytochemical Screening of Aqueous Stem Extract of A. Aspera

S.No.	Phytochemicals Tests	Aqueous Stem Extract of A. Aspera
1	Alkaloids	Presence
2	Tannins	Presence
3	Cardiac Glycoside	Absence
4	Steroids	Presence
5	Flavonids	Presence
6	Terpenoids	Presence
7	Phenols	Presence
8	Proteins	Absence
9	Saponins	Presence

Fourier transform infrared (FTIR) Spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy analysis, was used to identify the functional groups of the phytochemicals present in the A. aspera aqueous stem extract. The FTIR spectrum of Achyranthes aspera aqueous stem extract illustrated in (Figure 1), revealed strong peaks at 1400 cm^{-1} and 1638cm^{-1} indicates the presence of O-H inorganic carbonate stretch bond and N-H bending amine, along with C=C stretching alkene respectively, while 2112 cm^{-1} represents C≡C stretching of alkynes. The absorption bands at 3356cm^{-1} corresponds to the presence of phenol –OH group. When the FTIR spectra reports indicates the presence of the phytochemicals which acts a reducing/capping and stabilizing agent, which aid in the formation of nanoparticles AgNPs, CuNPs and Bimetallic Ag-CuNPs[14, 15].

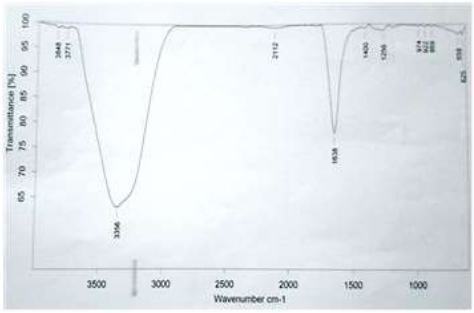


Figure 1: FTIR spectra of the Stem extract of A. aspera

Anti-bacterial activity

Anti-bacterial activity of Achyranthes aspera stem extracts and its synthesized nanoparticles was assessed by measuring the zone of inhibition(mm) in diameter around the wells containing various concentrations of nanoparticles of 25, 50, 75, 100 µL, studied against the bacterial pathogens of gram positive of Staphylococcus aureus and Streptococcus pneumonia, gram negative of Pseudomonas aeruginosa and E. coli by the Agar well-diffusion method [16-18]. The antibacterial activity of optimized AgNPs, CuNPs and Ag-CuNps was compared to a commercial antibiotic Ampicillin.

Table 2. Antimicrobial susceptibility profile of various extracts of Achyranthes aspera at different concentrations exhibited the ZOI in mm

S.No	Strain	Ampicillin				Stem extract				Silver nanoparticles				Copper nanoparticles				Silver-Copper nanoparticles			
		25 µL	50 µL	75 µL	100 µL	25 µL	50 µL	75 µL	100 µL	25 µL	50 µL	75 µL	100 µL	25 µL	50 µL	75 µL	100 µL	25 µL	50 µL	75 µL	100 µL
1	*Staphylococcus aureus*	23	26	27	29	16	20	22	24	12	17	18	21	18	18	19	23	18	21	24	27
2	*E.coli*	16	17	19	22	11	12	15	16	0	11	16	20	0	0	10	15	17	19	20	22
3	*Streptococcus pneumonia*	13	15	21	24	0	0	0	12	17	18	16	22	0	0	10	11	9	17	18	23
4	*Pseudomonas aeruginosa*	22	23	24	27	12	15	17	20	20	21	23	22	14	18	21	23	16	19	20	25

Anti-bacterial activity with Standard Ampicillin

In the present study Ampicillin used as standard antibiotic. At a concentration of 100µg, it exhibited the zone of inhibition (ZOI) of 29mm against Staphylococcus aureus and 22mm against E. coli. In addition, Streptococcus pneumonia and Pseudomonas aeruginosa showed the zone of inhibition 24mm and 27 mm, respectively (Figure 2).

Figure 2: Anti-Bacterial Activity of Standard Ampicillin **(A)** Staphylococcus aureus **(B)** E. coli **(C)** Streptococcus pneumonia **(D)** Pseudomonas aeruginosa

Anti-bacterial activity with Stem Extract

The stem extract of Achyranthes aspera also demonstrated anti-bacterial activity at a concentration of 100µg. It produced a ZOI of 24 mm against Staphylococcus aureus and 16 mm against Escherichia coli. Streptococcus pneumoniae and Pseudomonas aeruginosa showed inhibition zones of 12 mm and 20 mm, respectively, at the same concentration (Figure 3).

Figure 3: Anti-Bacterial Activity of Stem extract **(A)** Staphylococcus aureus **(B)** E. coli **(C)** Streptococcus pneumonia **(D)** Pseudomonas aeruginosa

Anti-bacterial activity of Silver Nanoparticles

The AgNPs synthesized from stem extract of Achyranthes aspera enhanced anti-bacterial activity at a concentration of 100µg, with a ZOI of 21 mm against Staphylococcus aureus and 20 mm against Escherichia coli. Streptococcus pneumoniae and Pseudomonas aeruginosa exhibited inhibition zones of 22 mm and 22 mm, respectively, at the same concentration (Figure 4).

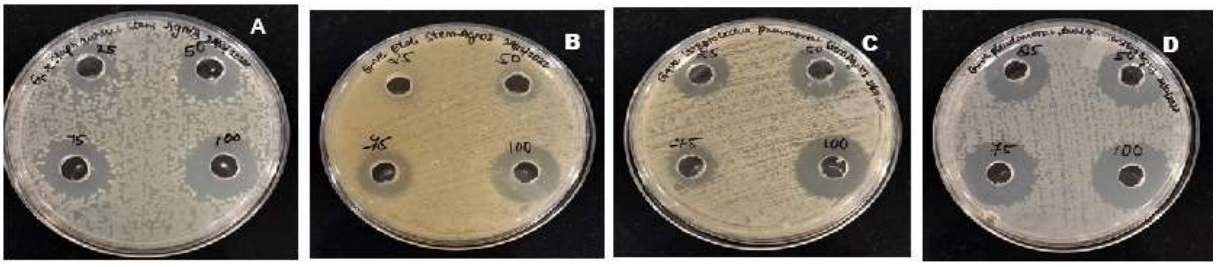


Figure 4: Anti-Bacterial Activity of Stem silver nanoparticles ($AgNP_S$) **(A)** Staphylococcus aureus **(B)** E. coli **(C)** Streptococcus pneumonia **(D)** Pseudomonas aeruginosa

Anti-bacterial activity of Copper Nanoparticles

The CuNPs synthesized from stem extract of Achyranthes aspera demonstrated a ZOI of 23 mm against Staphylococcus aureus and15 mm against Escherichia coli. Streptococcus pneumoniae and Pseudomonas aeruginosa showed inhibition zones of 11 mm and 23 mm, respectively, at the same concentration (Figure 5).

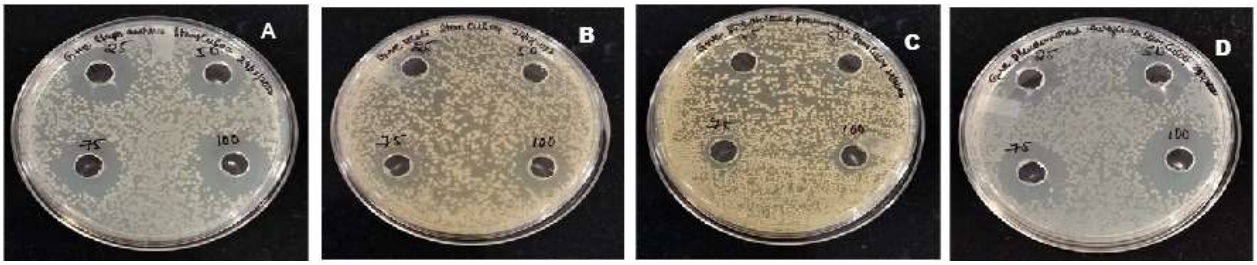


Figure 5: Anti-Bacterial Activity of Stem copper nanoparticles (CuNPs) **(A)** Staphylococcus aureus **(B)** E. coli **(C)** Streptococcus pneumonia **(D)** Pseudomonas aeruginosa

Anti-bacterial activity of Silver-Copper Nanoparticles

Bimetallic Ag-CuNPs synthesized using stem extract of Achyranthes aspera exhibited the highest anti-bacterial activity at a concentration of 100µg. They produce a ZOI of 29 mm against Staphylococcus aureus and 22 mm against Escherichia coli, while Streptococcus pneumoniae and Pseudomonas aeruginosa showed inhibition zones of 24 mm and 27 mm, respectively (Figure 6).

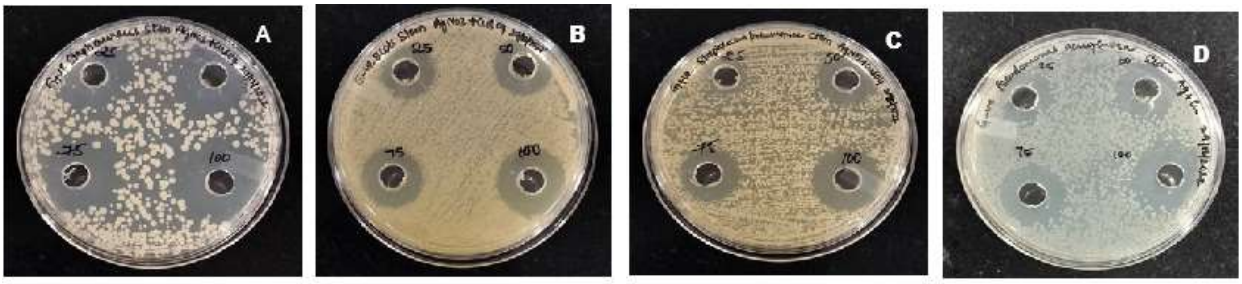
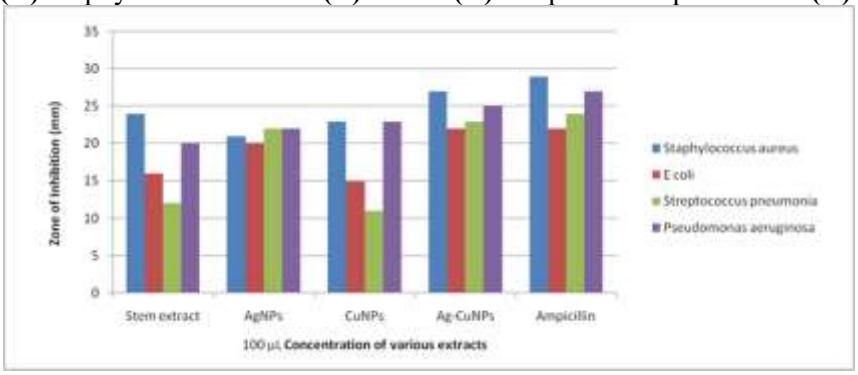


Figure 6: Anti-Bacterial Activity of Stem silver-copper nanoparticles (Ag-CuNPs).
(A) Staphylococcus aureus **(B)** E. coli **(C)** Streptococcus pneumonia **(D)** Pseudomonas aeruginosa



The Graph illustrates a comparative analysis of the antimicrobial activity of aqueous stem extract and synthesized nanoparticles against **(A)** Staphylococcus aureus **(B)** E. coli **(C)** Streptococcus pneumonia **(D)** Pseudomonas aeruginosa, with highest inhibition observed for the bimetallic Ag-CuNPs

Conclusion

The finding of the present study demonstrates the green synthesis of AgnPs, CuNPs, Bimetallic Ag-CuNPs using aqueous stem extract of Achyranthes aspera. The phytoconstituents present in the extract played a crucial role in capping/reducing and stabilizing the nanoparticles. The antimicrobial activity was evaluated against four pathogenic bacteria: Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, and Pseudomonas aeruginosa. Among the various extracts Bimetallic Ag-CuNPs showed a highest antibacterial efficacy. This implies the synergistic interaction between silver and copper ions in the bimetallic Nano formulation, enhancing their antimicrobial potential. The findings of this study highlights the promising application of green synthesized nanoparticle as an alternative to conventional antibiotics, especially in combating multi-drug resistant bacterial strains. Further studies are required to test the efficacy of the nanoparticles against wider range of microbial strains, including cytotoxicity assessments and in-vivo evaluations, are recommended to validate their potential for clinical and pharmaceutical use.

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Conflict of interest

Authors declare no conflict of interest related to this study.

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