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**A STUDY ON BIO SYNTHESIZED SILVER NANOPARTICLES AND ITS IMPACT
ON *S. aureus* AND *P. aeruginosa* GROWTH ISOLATED FROM CLINICAL
ISOLATES AND PROTON COUPLED MEMBRANE TRANSPORT**

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ABSTRACT

This study assessed the antimicrobial activity of biosynthesized AgNPs with an average size of 64 nm on two hospital strains of *S. aureus* and *Pseudomonas aeruginosa* resistant to a large number of antibiotics and a reference strain from a culture collection and a perspective for application of nanosilver with antibiotics to enhance antimicrobial activity. The prevalence of selected pathogens *S. aureus* and *P. aeruginosa* in 24 samples of wound swabs and ear infection samples submitted to the Microbiological Laboratory of Govt General Hospital, Guntur during the period Jan 2018-Oct 2018 was investigated. Results showed the potential of using prepared AgNPs along with selected antibiotics as an alternative to conventional antimicrobial agents that are currently used. Root-extract from *Achyranthes aspera* natural plant was used as a precursor for the synthesis of silver-nanoparticle material, which was characterised by a scanning electron microscopy having Energy Dispersion Spectroscopy (SEM + EDS), XRD for particle size determination, UV-Visible spectroscopy for stability studies, FT IR analysis for functional group determination. The main objective of this contribution was to prepare AgNPs from root extract and evaluate their antimicrobial activity on *S. aureus* and *P. aeruginosa* bacterial isolated from clinical samples considered as drug-resistant bacteria by minimum inhibitory concentration (MIC) method.

Keywords: Bio Synthesized Silver Nanoparticles, *S. aureus* and *P. aeruginosa*

1. INTRODUCTION

Antibiotic resistance of bacteria and other microorganisms is one of the most serious and grievous challenges of the twenty-first century. *Staphylococcus aureus* (*S. aureus*) is a facultative anaerobic, Gram-positive cocci. This bacterium is the most common cause of a wide variety of illnesses, such as impetigo, pimples, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, as well as life-threatening diseases, such as pneumonia, osteomyelitis, meningitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. It can affect almost any parts of the body, including skin, connective tissue, respiratory, bone, joint, and endovascular regions [1]. *P. aeruginosa* is associated with a strong increase in human infections [2]. They can cause opportunistic infections, especially in immunocompromised patients such as burn victims, patients with cancer, or those with cystic fibrosis. *Pseudomonas aeruginosa* has great intrinsic antimicrobial resistance limiting the number of effective antibiotics. Thus, other antimicrobial agents such as silver nanoparticles (AgNPs) are considered potential agents to help manage and prevent infections. AgNPs can be used in several applications against bacteria resistant to common antibiotics or even multiresistant bacteria such as *P.*

aeruginosa. Studies have demonstrated the effectiveness of AgNPs as dressings for covering burns to surgical devices and bone prostheses, and are incorporated into clothing – always with the aim of producing antimicrobial effect [3]. The multidrug resistance (MDR) observed in Gram-positive/negative bacteria are mostly attributed to over expression of efflux pumps and antibiotics-degrading enzymes. This MDR of *S. aureus* propels the search of new antibacterials with more efficiency and low toxicity. Antimicrobial resistances of bacterial pathogens are a major problem for the treatment of animal and human patients with bacterial diseases. Determination of MIC of antibiotics for bacteria plays a crucial role for determination of antibiotic resistance of bacteria. *P. aeruginosa* shows high intrinsic resistance to antibiotics and has an extraordinary capacity to acquire new resistance mechanisms [4]. Relatively little data exist so far about the faecal colonisation by *P. aeruginosa* isolates in healthy humans, as well as about their antimicrobial resistance and virulence characteristics [5]. Children tend to be exposed to more disease-causing bacteria through diary activities such as childcare and mouthing behaviours; they are more vulnerable to bacterial illness than adults,

and they develop many nonfatal bacterial infections that require antimicrobial treatments, whereas no descriptions exist about the faecal carriage and characterisation of *P. aeruginosa* isolates from children. Currently, the unique antimicrobial properties of AgNPs have led to their application in areas such as clothing manufacturing, food preservation, and water purification [6]. More importantly, AgNPs are being increasingly utilized in the medical industry due to their antibacterial, antifungal, antiviral, anti-inflammatory, and osteoinductive effects as well as their ability to enhance wound healing [7].

Plant kingdom contains a variety of pharmacologically active secondary metabolites, and some of them have been reported for their antibacterial activities. Their use to combat *S. aureus* and *Pseudomonas aeruginosa* antibiotic resistance is an attractive strategy. In regard to the loss of efficacy of several antibiotics and the scarcity of new antibacterial agents, it is also important to search for substances capable of restoring the activity of antibiotics [8]. Antibacterial screenings of Indian plants have yielded promising results in the past. The present study was set up to evaluate the antisbiotic potential of *Achyranthes aspera* root extract based silver nanoparticles. One of the many plants used is *Achyranthus aspera*. *A.*

aspera Linn. belongs to the family Amaranthaceae, is an annual, stiff erect or procumbent, annual or perennial herb, 1-2m in height, often with a woody base, commonly found as a weed of waysides, on roadsides. The plant is used in indigenous structure of traditional medicine systems as an antibacterial, antiviral, anticancer, antioxidant, anti-inflammatory and antiarthritic, anti-fertility, and antiplasmodic [9]. The juice extracted from the root of this plant is mixed along with the root extracts of *Urena lobata* and the bark of *Psidium guajava*, and is used in the treatment of diarrhoea and dysentery [10]. The major phytochemicals isolated from the plant species is used for the treatment of kidney stone problems [11]. The plant-mediated green synthesis of NPs is gaining importance due to its eco-friendliness and simplicity. Recently, using the leaf extracts of *A. aspera* synthesize of Ag and AuNPs nanoparticles was reported [12]. The present research work was undertaken for determining resistance of *Staphylococcus aureus* & *Pseudomonas aeruginosa* against commonly used antibiotics and along with Biologically synthesized nanoparticles. The objective of this study is to characterize the pyogenic bacteria *S.Aureus* and *Pseudomonas aeruginosa* from wound swabs and ear infection samples to determine their antibiotic susceptibilities along with AgNPs

to various generations of antibiotics commonly used in chemotherapeutic interventions.

2. Experimental

2.1 Synthesis of AgNPs from *Achyranthes aspera* root extract

Achyranthes aspera plant dried root material in bulk (~1 kg) was collected and roots were cut in to small sizes, washed thoroughly in running tap water, followed by distilled water, and then shade further dried for 10 days (Figure 1). The dried root material of *Achyranthes aspera* was porously powdered using mechanical blender. The porously powdered root material was subjected for soxhlet extraction. The dried, powdered roots of

Achyranthes aspera (400g) were extracted using n-hexane (2L) in soxhlet apparatus for 72 hrs. The extractions were carried out until the solvent become colourless in the timble tube of soxhlet. These extracts were carried out by the above mentioned procedure for further analysis. All the fractions of extracts were evaporated using rotatory flash evaporator and vacuum dried. In a typical reaction procedure, 10 ml (Ethanollic) of root extract was added to 90 ml of 10^{-3} (M) aqueous silver nitrate solution in to four flasks each. The flasks (aqueous) were then incubated at room temperature for different time intervals to identify the color changes and to study the stability of obtained AgNPs.



Figure 1: *Achyranthes aspera* (a) plant, (b) roots (c) root powder

2.2 AgNPs Characterization

The reduction of silver ions in the colloidal solution was confirmed by UV-Visible spectroscopy. A small aliquot from Ag NPs was taken in a quartz cuvette and observed for wavelength scanning between 200 to 900 nm with distilled water as a reference. The UV-Vis absorption spectrum of the sample was performed in Perkin Elmer Spectrophotometer, at different time

2hrs, 12hrs, 24 hrs and 48 hrs min after addition of Achyranthus root extract on AgNO_3 solution. Infrared spectra (IR) were obtained with a FTIR spectrophotometer (IRAffinity, Shimadzu) from 400 to 4000 cm^{-1} . The SEM images and the elemental analysis were recorded using the Scanning Electron Microscopy (SEM) model FEI Quanta 200 FEG high microscopy resolution with EDAX Energy

Dispersive Spectroscopy (EDS) and X-ray diffraction measurements were carried out on the Philips Xpert pro XRD system (DY 1650)

2.3 Bacterial strains collection

Twenty four (n=24) samples of ear infection and wound swabs of patients (Table 1) attended to government general hospital during the period of March 2018 to October 2018 with prior permission of patients and followed by ethical guidelines used for this study. Specimens of ear discharge were collected using sterile cotton swab sticks by the assistance of medical officer. Wounds swabs (WS) were collected using sterile swabs sticks.

2.4 Isolation Procedures

Disc diffusion method was used to assess antimicrobial activities of synthesized silver nanoparticles against *S. aureus* and *P. aeruginosa* isolated from wound and ear secretions were sub culture and incubated at 37°C for 24 h. Fresh cultures were taken and spread on Mackonkey agar (HiMedia) plates to cultivate bacteria. Sterile paper discs of 5 mm diameter were saturated with double distilled water (as control), plant extract and silver nanoparticles solution were placed in each agar plate and incubated again at 37°C for 24 hrs. Based on inhibition zones around the discs, the antimicrobial activities were measured

saturated with plant extract and synthesized silver nanoparticle.

2.5 Characterization of Isolates

Characterization of isolates was carried out by employing macroscopic, microscopic, physiological, and serological and biochemical tests.

2.6 Ethical Statement

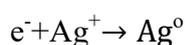
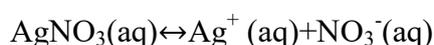
Ethical clearance was obtained from the ethical committee, College of Medicine and Health Sciences, GGH, Guntur. Official permission and written informed consent were obtained from Hospital administration office and from each study participant, respectively. The assent of children (<18 years old) was obtained from their family or guardian. All the information obtained from each study participant was kept confidential. The laboratory result from the study participant was communicated to their doctors for appropriate treatment.

3.0 RESULTS AND DISCUSSION

3.1 UV Visible spectroscopic studies

After the addition of the *Achyranthus* root extract to the aqueous AgNO₃ solution, light yellow brown colors slowly appeared in the mixture, indicating the formation of Ag-NP and finally turn into dark brown color Figure 2. The characterization absorption spectra was the important properties of the Ag NPs, and the UV-Vis spectra was a good method for characterization of the formation and

growth of Ag NPs. Absorption peaks of silver nanoparticles synthesized using green tea extract solutions were obtained at wavelength of 430 nm (Figure 2). A change in color occurred because of excitation in surface Plasmon resonance wherein, it can be an indication of the formation of Ag-NP [13]. The UV-Vis spectra showed maximum absorbance at 430 nm, which increased with time of incubation of silver nitrate with the plants extract. The observation indicated that the reduction of the Ag^+ ions took place extracellular. It was reported earlier that absorbance at around 436 nm for silver is a characteristic of these noble metal particles [14]. The bioreduction of Ag^+ ions was monitored by periodic sampling by the UV spectrophotometer. The AgNPs in the freeze-drying bottle were suspended in ultrahigh purity water for all characterization methods and antibacterial assays. During biosynthesis of silver nanoparticles when stem extract was added to 100 ml of 1 mM AgNO_3 salt, the ionization took place as follows:



It is assumed that the silver ions enter inside the plant cell via the H^+ ATPase protein embedded in the thylakoid membrane by an electrogenic pump. Synthesis of silver nanoparticles is a photochemical reduction reaction.

3.2 FT IR analysis

The bioreduction and stabilization of the as-synthesized Ag-NPs could be credited to the presence of $-\text{OH}$, $\text{C}-\text{C}-\text{H}$, and the $-\text{C}=\text{O}$ functional group of the amide and synergistic effect of other functional groups earlier highlighted. Earlier reports had indicated the ability of protein moiety (amines, amides and peptides) to bind themselves to nanoparticles surfaces thereby acting as capping agents to prevent aggregation.

FTIR spectroscopy was used to ascertain the biomolecules and functional groups that are responsible for the reduction and capping of the as formed AgNPs. FTIR spectra of the root extract in figure 3 demonstrate the existence of numerous functional groups. The peak at $3445.2\text{cm}^{-1}/3442\text{cm}^{-1}$ shows $-\text{OH}$ stretch, the peaks at $2961.5/2959.4\text{cm}^{-1}$ designates methylene $-\text{C}-\text{H}$ asymmetric respectively, the peak at 1638.2cm^{-1} reveals the presence of carbonyl peak, the peak at 1456cm^{-1} indicates to methylene $\text{C}-\text{H}$ bend, 1242cm^{-1} is showing $-\text{OH}$ in plane, 1074cm^{-1} is an evidence of skeletal $\text{C}-\text{C}$ vibrations, 740cm^{-1} is indication of aromatic $\text{C}-\text{H}$ out of plane bend. Some of these functional groups are responsible for the reduction of silver salt to silver nanoparticles as illustrated in the FTIR spectra of our silver nanoparticles in figure 3. When compare the two spectra of

before and after formation of AgNPs, all peaks exists at same wave number (cm^{-1}) with little intensity change indicates that there is no chemical reaction between natural products present in root extract and silver ions. Formation of AgNPs is expected to be a physical reaction only.

3.3 SEM-EDS analysis

The surface morphology, size and shape of the silver nanoparticles were analyzed by Scanning Electron Microscope. Figure 4 shows the SEM image of silver nanoparticles synthesized from root derived extracts. The SEM images show individual silver nanoparticles which are predominantly spherical in shape as well as number of aggregates with no defined morphology. The presences of biomolecules in the root extracts has resulted in the synthesis of spherical silver nanoparticles and the aggregation may be due to the presence of secondary metabolites in the root extracts. The SEM image shows the size of the silver nanoparticles ranging from 40 to 50 nm. An X-ray energy dispersive spectroscopy (EDS) study was carried out to confirm the formation of silver nanoparticles. EDS peaks corresponding to element silver show the presence of silver as the ingredient element and the formation and purity of silver nanoparticles synthesized from leaf extract and callus extracts (Figure 4). The sharp peak in the silver region was

observed at 3 keV confirming the presence of silver nanoparticles due to the Surface Plasmon Resonance [15]. Generally silver nanocrystals demonstrate typical optical absorption peak approximately at 3 keV due to Surface Plasmon Resonance [16].

The EDS elemental analysis of the synthesized silver nanoparticles showed highest proportion of silver followed by C and O. The weak oxygen signal may be due to X-ray emission from carbohydrates/proteins/enzymes present within the extracts [17] or possibility of silver oxide nanoparticles formation after synthesis of silver nanoparticles, which reacts with water in the solution since the nanoparticles are highly reactive due to their high surface to volume ratio [18].

3.4 X-Ray Diffraction

Furthermore, evidence for biosynthesis of silver and copper nanoparticles was confirmed by the X-ray diffraction (XRD) analysis (Figure 5). The X-ray diffraction (XRD) analysis explained the structure of silver nanoparticles. In Figure 4 XRD patterns for silver nanoparticles between 2θ of $20-80^\circ$ are shown. It is indexed by JCPDS card no (1257-5). XRD patterns show the diffraction peaks of silver nanoparticles at 2θ values for 32.7° , 35.8° , 41.9° , 61.8° , and 74.4° which matched with 110, 111, 200, 220, and 311 lattice planes of face centered cubic structure, respectively [19].

XRD confirmed that silver nanoparticles are crystalline in nature with the FCC structure. Average crystalline size calculated by the Debye Scherer equation is 67.4 nm. The average size of synthesized AgNPs was calculated by using the Debye Scherer equation is correlated with that obtained from SEM images.

3.5 Antibacterial study

Patients: A total of 24 study participants with wound or Ear infection were included in the study. Among these, 7 (29.1%) are women with an average age of 34.5years, four (16.6%) women suffering with ear infection, three (12.5%) from wound infection. 12 (50%) men patients with an average age of 36 years, 8 (33.3%) men suffering with ear infection, 4 (16.6%) from wound infection, third category in this study is five children with an average age of 14.2years suffering with ear infection (2 (8.33%) and 3 (12.5%) suffering with wound infection (Table1).

3.6 Bacterial identification

Of the 24 wound and ear samples collected from different category of patients in the hospital, 17 samples (70.83%) showed bacterial growth after 48 hours of incubation whereas 4 samples (16.6%) were negative for growth. Based on Gram staining, morphological features, culture characteristics, and biochemical characterization, the bacterial isolates were assigned to two bacterial species.

Staphylococcus aureus was the most frequent pathogen as revealed by 70.3% occurrence followed by *Pseudomonas aeruginosa* (45.8%) (Table2). Our findings correlate with Zhang et al.[20] who reported predominance of *S. aureus* and *P. aeruginosa* in pus samples from patients with severe intra-abdominal infection. In another study, *S. aureus* was the dominant bacterial species from wounds followed by *P. aeruginosa* [21]. Details information of isolates in the ear and wound samples were given in Table 2.

3.7 Antibiotic/AgNPs sensitivity of bacterial isolates

Antibiogram results from the present study show that *P. aeruginosa* & *S. aureus* isolated three categories were more resistant in the following order: Plant root extract>Ciprofloxacin (CF)>Norfloxacin (NF)>AgNPs>CF-AgNPs>NF-AgNPs (Table 1). *P. aeruginosa* from ear sample was more susceptible to tested antibiotics+ AgNPs, compared to AgNPs and antibiotics alone, both species showed more susceptible to AgNPs+Antibiotics. Unlike some reports in which Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) was associated with wound infections[22], our findings revealed susceptibility in *S. aureus* isolates towards AgNPs, and in combination with Ciprofloxacin (CF), Norfloxacin (NF), however, exhibited minimal resistance and

were susceptible to most of the antibiotics+AgNPs(Table 3). Both Gram-positive isolates were fully susceptible to antibiotics+ AgNPs. The reason may be chronic wounds tending to show monomicrobial infections. The present study revealed polymicrobial infections, mainly by *P. aeruginosa* and *S. aureus*, which is consistent with report from India [23]. This study intended to gain new insights into the *P. aeruginosa* in non-clinical environments, analysing the occurrence, and characteristics of *P. aeruginosa* from samples of children/Men/Women. In fact, the occurrence of *P. aeruginosa* isolates in this study was in the range detected in other studies [23]. The presence of *P. aeruginosa* in stool constitutes a digestive reservoir of the bacteria that may be of importance in *P. aeruginosa* pathophysiology.

AgNPs have been widely used as an antibacterial coat in medical applications

such as wound dressings, cardiovascular implants, catheters, orthopedic implants, and dental composites. Silver wound dressings have been used for over a decade to clinically treat various wounds, such as burns, chronic ulcers, toxic epidermal necrolysis, and pemphigus [24-25].

Effects of concentrated Ag nanoparticles colloid solution on bacterial proton-coupled membrane transport and ATPase activity

As in the case of *P. aeruginosa* & *S. aureus* bacterial growth was detected only in the case of 1:100 and 1:200 dilutions of Ag nanoparticles, proton-coupled membrane transport and ATPase activity was measured in the presence of these concentrations. It observed that in the case of 1:100 dilutions the ATPase activity was lowered by 15 fold compared to the control sample.

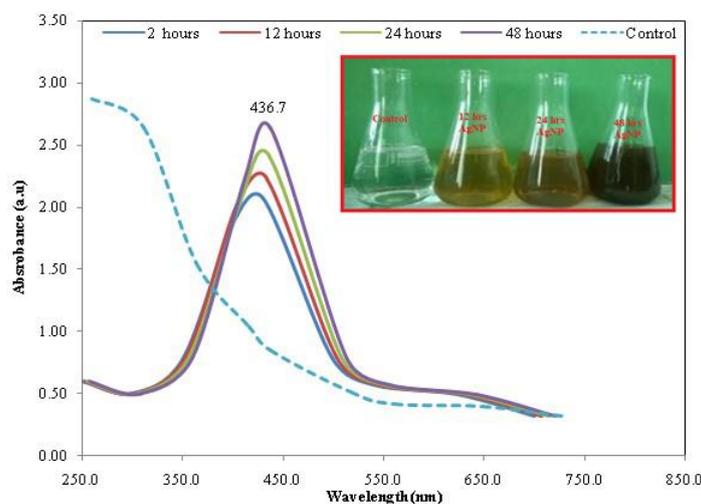


Figure 2: Silver nanoparticles before (control) and after bio reduction (Ag^+ to Ag^0) –UV Visible absorption spectra of SNPs after bio reduction (inset colour change during reduction process at different time intervals)

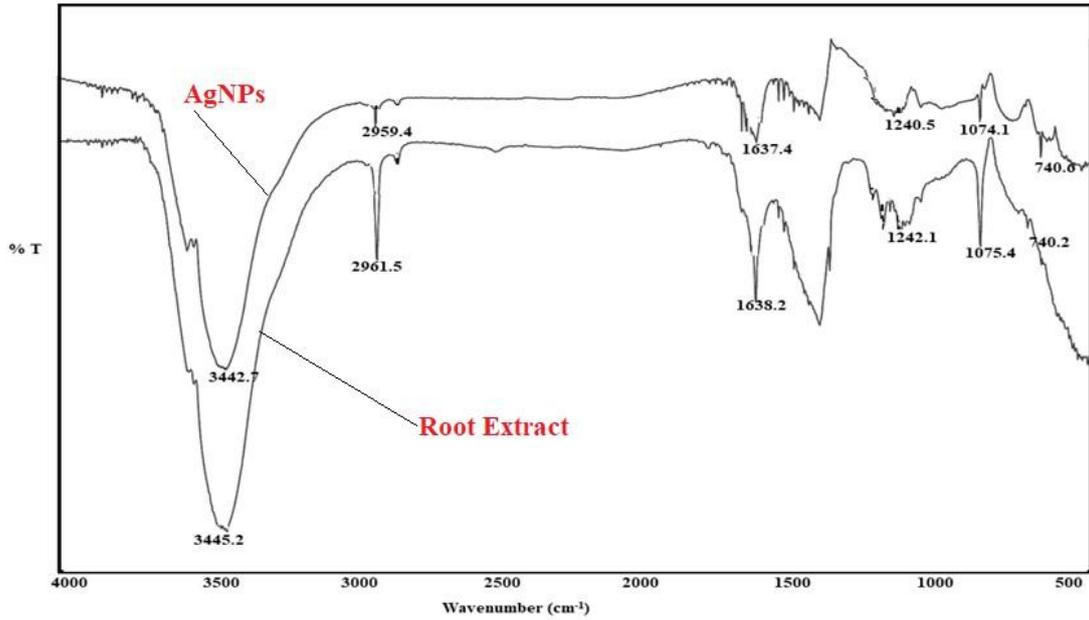


Figure 3: FT IR Spectra of root extract and AgNPs derived from *Achyranthes aspera* root extract

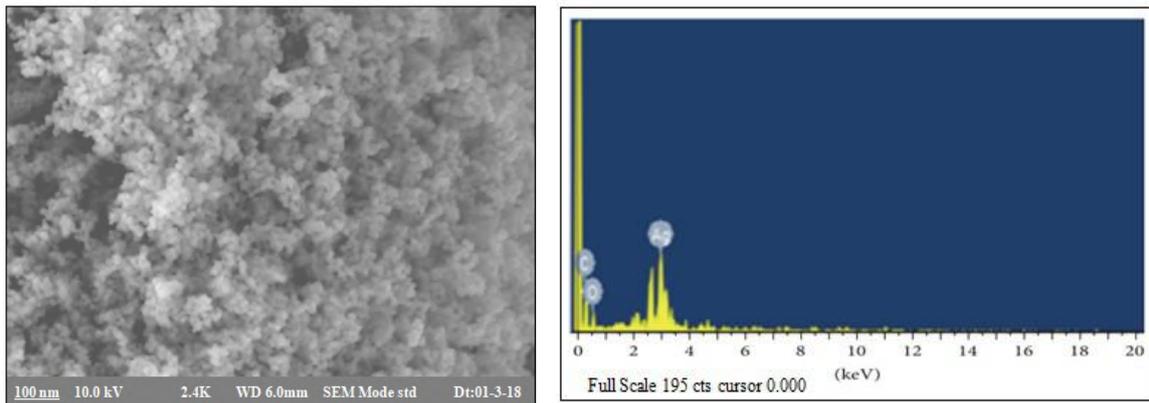


Figure 4: SEM (left) -EDX spectra (right) of AgNPs derived from *Achyranthes aspera* root extract

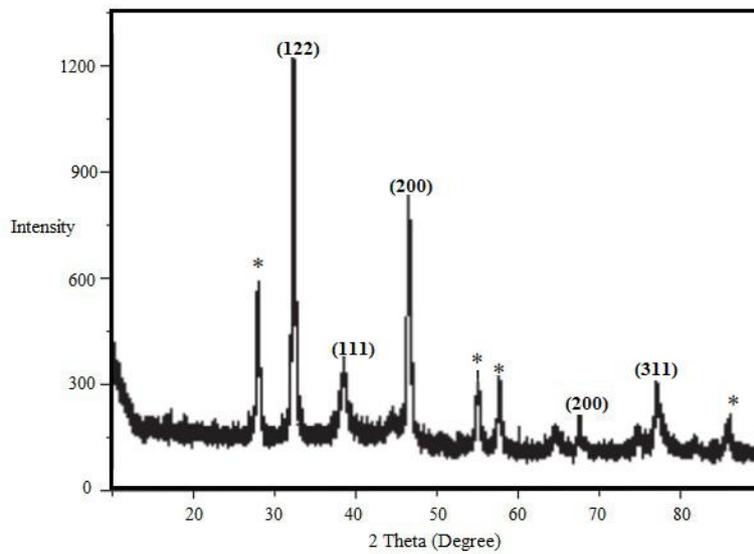


Figure 5: XRD spectra of AgNPs derived from *Achyranthes aspera* root extract

Table 1: Source and number of different samples used

Sample Name	Women -Average age (34.5Yrs)	Men -Average age (36Yrs)	Children -Average age (14.2Yrs)
Ear Swab	4	8	2
Wound Swab	3	4	3
Total (n=24)	7	12	5

Table 2: Distribution of different pathogens

Sample Name	Sample Source	<i>S. aureus</i>	<i>P. aeruginosa</i>
Ear Swab	Women	3 (75%)	2 (50%)
	Men	6(75%)	3 (37.5%)
	Children	1(50%)	2 (100%)
Wound Swab	Women	2(66.7%)	2 (66.6%)
	Men	3 (75%)	2 (50%)
	Children	2(66.7%)	Nil

Table 3: Antibiotic susceptibilities of *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria isolated from wound and ear samples at Givr Gen.Hospital Guntur against root extract, Nanoparticles, along with antibiotics and general antibiotics combination with AgNPs

Name of the Bacteria	Source	Antibiotic sensitivity (%) of bacterial isolates					NF-Ag NPs
		Root Extract	Ciprofloxacin (CF)	Norfloxacin (NF)	Ag NPs	CF-Ag NPs	
<i>Pseudomonas aeruginosa</i>	Wound (n=4)	24.10	29.88	27.19	83.77	86.36	97.90
	Ear (n=7)	13.70	16.99	32.96	78.96	81.40	84.66
<i>Staphylococcus aureus</i>	Wound (n=10)	37.60	46.62	11.19	83.21	85.79	89.41
	Ear (n=7)	20.70	25.67	9.50	84.65	87.27	89.27

4. CONCLUSION

An increasing attention towards green chemistry and utilization of plant extracts for metal nanoparticles synthesis lead to the development of environment-friendly techniques. A benefit of silver nanoparticles synthesis by using plant extracts is that it's economical, energy efficient and cost-effective, provides healthier workplaces, and protects human health and environment. Green synthesized silver nanoparticles play a significant role in the area of nanotechnology. Synthesis of nanoparticles using plants has several advantages over other biological organisms

which overcome the time-consuming process of growing microbial cultures and maintenance. Silver nanoparticles have been successfully synthesized via a green synthesis method with *Achyranthus* root extract. The synthesized nanoparticles have a size range between 60-68.4nm. XRD and UV-Vis analysis confirmed the formation of silver the nanoparticles .The amount of silver nanoparticles formed increased with increase in reaction time and increase in temperature. The synthesized nanoparticles have potential application in waste water treatment, bio-medicals, medical textiles, wound dressing, antimicrobial applications,

etc. The simple procedure used in the synthesis of the silver nanoparticles in this study has the advantage of large scale production as it can be used efficiently in large scale production. The current study concluded that *Achyranthus root* extract based synthesized Ag NPs revealed significant inhibitory action and has great potential as antimicrobial compound against tested pathogens because of the tested resistant bacteria to an antibiotic, established their vulnerability to antibiotics combined with Ag-NPs. Though, production of nanoparticles may strongly terminate the chemical agent's problem, which has possible side effects against its application.

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