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Green synthesis of iron, copper and silver nanoparticles and their antibacterial activity on animal pathogens – A Comparative study

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Abstract— Prevalence of antibiotic resistance in the animal pathogens is posing a threat to the treatment of various veterinary diseases like anthrax, brucellosis, gastritis caused by *Bacillus anthracis, Brucella and Staphylococcus aureus.* Antibiotic resistant bacteria may spread from animals to humans through various veterinary products. Challenges in the area of antibiotic resistance are met not only by combined drug therapy and the use of novel antimicrobial agents, but also the use of nanomedicine is offering an alternative to treat drug resistant pathogens. Nanoparticle research is currently an area of intense scientific research due to their unique properties like smaller size, penetrating ability into the cell and diversified applications in fields like biomedicine, agriculture, information technology. Since nanoparticles serve as potent antimicrobial agents, the present study mainly focuses on evaluating the efficacy of Iron, Copper and Silver nanoparticles as antimicrobial agents on animal pathogens. Green synthesis of nanoparticles from plant sources is the most emerging method as compared to the chemical and physical methods as it is less time consuming, cost effective and eco- friendly.

Neem (*Azadirachta indica*) leaf extract was used to synthesis the Iron, Copper and Silver nanoparticles and were further characterized using techniques like UV-Vis Spectroscopy and SEM (Scanning Electron Microscopy). The synthesized nanoparticles were tested for antibacterial activity using well-diffusion assay against *Escherichia coli (E.coli*) and *Staphylococcus aureus (S.aureus)* isolates obtained from P. V. Narsimha Rao State Veterinary University. Antibacterial study reveals that Silver nanoparticles synthesized using Neem leaf extract show significant antibacterial effect when compared to Iron and Copper nanoparticles.

Keywords*: green synthesis, Azadirachta indica, nanoparticles, animal pathogens, antibiotic resistance, antimicrobial activity, well-diffusion method.*

I. INTRODUCTION

Green synthesis of nanoparticles provides a rapid metallic nanoparticle production by offering high yield, low cost, eco-friendly, non-toxic, simple and reproducible approach when compared to physical and chemical methods [1]. There are many physical and chemical methods for the synthesis of nanoparticles which involves harmful chemicals, low material conversions and high energy requirements. To avoid the use of harmful chemicals and high energy demands, biological approach appears to be very appropriate for the synthesis of nanoparticles. Biological synthesis includes the use of natural materials like plants, bacteria, fungi [2]. Nanoparticles produced with the use of plants are found to be more stable and rate of synthesis is faster when compared to those produced by microorganisms [3]. Plants like Neem, Aloe Vera and Tulsi serve as an important source for the synthesis of metallic nanoparticles. Neem products are found to be anthelmintic, antiparasitic, antibacterial and antifungal [4].

Treating bacterial infections in humans as well as in animals is increasingly complicated due to the ability of pathogens to develop resistance to available antibiotics. The efficacy of antibiotics is becoming endangered due to the emergence of antibiotic resistance by bacteria [5]. Drug resistant bacteria like *Escherichia coli, Staphylococcus aureus, Enterococcus sps and Klebsiella sps* are becoming more and more common. These resistant bacteria can spread from individual to individual. To overcome the antibiotic resistance, novel strategies like use of natural antimicrobials, combination of synergistic therapy and metal nanoparticles can become a successful approach [2].

The present study focuses on the green synthesis of Iron, Copper and Silver nanoparticles using neem (*Azadirachta indica*) leaf extract and testing and comparing their antibacterial activity against animal pathogens like *Escherichia coli* and *Staphylococcus aureus* as these nanoparticles have various biomedical applications in drug delivery, treating cancer, inhibiting the bacterial growth [6-8].

Treatment of veterinary diseases is a great challenge because of multidrug resistant bacteria. This antibiotic resistance has resulted in the development of new methods for dealing with the treatment of infections caused by these multidrug resistant bacteria [9]. These new methods include the development of nanostructured materials to deal with the antibiotic resistance. Nanoparticles interfere with the bacterial drug resistance mechanisms and inhibiting their growth [10]. The recent area of nanotechnology is focusing more on the green synthesis of nanoparticles than the physical or chemical synthesis as green synthesized nanoparticles are more stable, non-toxic and eco-friendly. A study reveals that the significant effect of combined therapy of silver nanoparticles and antibiotics on the veterinary bacteria [11]. When Iron nanoparticles produced using Musa ornata flower showed significant antibacterial activity against *S.aureus* and *E.coli* [12].

II. METHODOLOGY

Collection of Neem leaves:
From Neem (*Azadirachta indica*) tree, fresh leaves were collected from campus of Bhavan's Vivekananda Degree College, Sainikpuri.

Preparation of Neem leaves extract:
The fresh Neem leaves of 200gm were taken washed twice with the distilled water and these washed leaves were subjected to drying for 24 hours. After drying, the leaves were cut into small pieces and one liter of distilled water was added and boiled for 30 minutes. After cooling, the solution was filtered using the Whatman No. 1 filter paper. The filtrate of leaf extract was collected in a beaker and was stored at 4℃ for further use [13].

Preparation of reagents:
Ferric chloride ($FeCl_3$) solution:
1M solution of $FeCl_3$ solution was prepared and labelled it as stock. Different concentrations (1mM, 2mM, 10mM and 20mM) of $FeCl_3$ were prepared from the stock solution [13].

Copper sulphate ($CuSO_4$) solution:
1M solution of $CuSO_4$ solution was prepared and labelled it as stock. Different concentrations (1mM, 2mM, 10mM and 20mM) of $CuSO_4$ were prepared from the stock solution [14].

Silver Nitrate ($AgNO_3$) solution:
1M solution of $AgNO_3$ was prepared and labelled as stock. From the stock solution, different concentrations (1mM, 2.5mM, 5mM, 10mMand 20mM) of $AgNO_3$ solutions were prepared [15].

Green synthesis of nanoparticles:
90 ml of different concentrations range of $FeCl_3$ (10mM-20mM), $CuSO_4$ (1mM - 20mM) and $AgNO_3$ (1mM- 20mM) solutions were added to the 10 ml of plant extract respectively. The solutions were incubated overnight at room temperature under dark conditions. After incubation the colour change was observed from orange to black for iron nanoparticles, blue to green for copper nanoparticles and yellow to brown colour for silver nanoparticles.

Characterization of nanoparticles:
The nanoparticles were characterized using UV-Vis spectroscopy [16] and SEM [17].

Bacterial cultures and identification studies:
Collection of animal pathogens:
Bacterial cultures were obtained from P.V. Narsimha Rao State Veterinary University. The cultures used for study were 4 strains of *E.coli* causing diarrhoea in sheep and 3 strains of *S. aureus* causing gastritis in buffalo and were sub-cultured on EMB (Eosin Methylene Blue) agar and MSA (Mannitol Salt Agar).

Identification of bacterial cultures:
The obtained cultures were identified microscopically by Gram staining [18] and biochemically by IMVIC (Indole, Methyl red, Voges Proskauer and Citrate) tests [19]. The cultures were also tested for antibiotic sensitivity and resistance using Disc diffusion method [20].

Antibacterial assays:
The Neem plant extract and nanoparticles synthesized using Neem plant extract were tested for their antibacterial efficiency against animal pathogens using Agar well assay [21].

III. RESULTS AND DISCUSSION

Collection of bacterial cultures and identification studies:
Seven bacterial animal pathogens were obtained from P. V. Narsimha Rao State Veterinary University and were used for antibiotic resistance studies. The collected bacterial cultures were identified culturally, microscopically by Gram staining and biochemically by IMVIC tests. The growth of bacterial colonies on nutrient agar was studied. Two types of colonies were noted. One as pin pointed colonies and other type of colonies as golden yellow colonies. Pin pointed colonies when cultured on Eosin Methylene Blue agar medium (EMB), gave metallic sheen colonies. Microscopic studies indicates that four bacterial cultures with metallic sheen on EMB agar medium were found to be Gram negative (Figure 1) and three bacterial cultures with golden yellow colonies on nutrient agar medium were found to be Gram positive (Figure 2). Biochemically, all the Gram negative strains were found to be Indole, Methyl red positive and Voges Proskauer, Citrate utilization test negative. Hence they could be *E.coli* strains and were designated as E1, E2, E3 and E4. All the Gram positive strains were found to be Methyl red

positive and Indole, Voges Proskauer and Citrate negative. Hence these strains could be *S.aureus* and were designated as S1, S2 and S3. The IMVIC results were summarized in the table.1.

Antibiotic sensitivity studies:

Antibiotic resistant infections are already widespread all across the globe, particularly Gram positive strains of resistant *S.aureus* and Enterococcus species are posing a biggest threat (20). Even animal pathogens like *E.coli* and *S.aureus* of buffalo and sheep are drug resistant. Therefore the present study is carried out to assess the drug resistance against various antibiotics. All the strains of *E.coli* (E1, E2, E3 and E4) and *S.aureus* (S1, S2 and S3) were tested for antibiotic sensitivity. The antibiotics used for testing were Trimethoprim, Tetracycline and Penicillin G. The strain E1 is resistant to all the 3 antibiotics. The strains E2 and E4 strains were found to be resistant to Tetracycline and Penicillin G whereas strain E3 was resistant to Penicillin G (Figure 3). The strain S1 was found to be resistant to Tetracycline (Figure 4, Figure 5). However sensitivity was found at very high concentrations of antibiotics.

Antibacterial activity of plant extract:

Since the pathogens were found to be resistant to most of the antibiotics, the plant products were evaluated for antimicrobial activity. As the Neem tree has important medicinal value, the leaf extract of Neem was used for antimicrobial activities against antibiotic resistant pathogens. However, the present study indicated that all the seven pathogens were found to be resistant to the Neem leaf extract (Figure 6). Similar result was found with Neem leaf extract against *S.aureus* from animal origin (22). Hence the plant mediated biosynthesized nanoparticles (FeNPs, CuNPs and AgNPs) were focused to challenge the concept of drug resistance.

Green synthesis of Iron, Copper and Silver nanoparticles:

The use of green synthesized nanoparticles represents a suitable and alternative strategy for treating infections caused by drug resistant bacteria (23). The plant mediated Iron, Copper and Silver nanoparticles as antimicrobial agents are advantageous to use over traditional and conventional antimicrobial agents. The Iron, Copper and Silver nanoparticles were synthesized using Neem leaf extract and were tested for antimicrobial activity against drug resistant animal pathogens *E.coli* and *S.aureus.* Such green synthesized nanoparticles of Iron, Copper and Silver were not only cost effective but also eco-friendly over the chemically synthesized nanoparticles. FeNPs, CuNPs and AgNPs were further characterized using UV-Vis spectroscopy and SEM analysis. The colour change from yellow to brown after the addition of plant extract to $AgNO_3$ solution is considered as an indication of the formation of silver nanoparticles. These colour intensities recorded using UV-Vis spectrophotometer between 405nm-445nm. An extreme absorption was detected at 425nm (Graph.2) after 24 hours of incubation. Similarly the absorption maxima for Iron and Copper were detected at 260nm and 340nm respectively (Figure 7). Iron nanoparticles synthesized from *Azadirachta indica*, Copper nanoparticles synthesized from Punica granatum were found to show absorption maxima at 268nm and 420 nm respectively (24,25). The sizes of synthesized nanoparticles by SEM analysis were found to be 58.9-78.0nm for iron nanoparticles (Figure 8) the size of Iron nanoparticles was found to be 58-79nm when produced from *Glycosmis mauritiana* (6), 49.6-95.4nm for copper nanoparticles (Figure 9) but when Copper nanoparticles produced from *Punica granatum* were found to be of 56-59nm in size (24) and 63.6-88.8nm for silver nanoparticles (Figure 10) respectively and the size was found to be 20-40nm when Silver nanoparticles produced from Mulberry leaf extract (25). The pictures showed that the particles were of spherical shape.

Antibacterial activity of synthesized metal nanoparticles:

The synthesized Fe, Cu, Ag nanoparticles were significantly screened for the antibacterial activity against 7 bacterial cultures. The antibacterial activity was determined by agar well assay. The growth of *E.coli* E1, E2, E3 and E4 strains was inhibited by Silver nanoparticles of 20mM. However, strain *E.coli* E4 was inhibited also by Copper nanoparticles of concentration 10mM. Iron nanoparticles did not show any effect on all the strains of *E.coli* (Figure 11). Similarly the growth of *S.aureus* S1, S2 and S3 strains were inhibited by Silver nanoparticles, Copper nanoparticles and Iron nanoparticles of 20mM concentration. However, Iron nanoparticles did not show any effect on the *S.aureus* S2 strain (Figure 12). Effect of Iron, Silver and Copper nanoparticles were studied by measuring the zones of inhibition of growth of *E.coli* (E1, E2, E3 and E4) and *S.aureus* (S1, S2, S3). The maximum zone of inhibitions of growth were found with Silver nanoparticles indicating the bacterial cultures were more sensitive to Silver nanoparticles when compared with Iron and Copper nanoparticles (Figure 13, 14, 15) Similar inhibition was found with the Silver nanoparticles against *P.aeruginosa* and *S.aureus* which were isolated from mastitis infected goat (23). Whereas iron nanoparticles produced from *Gardenia jasminoides* were found to be potent against *S.aureus* with zone of inhibition 16mm and for *Lawsonia inermis*, it was 15mm. (26). Similarly copper nanoparticles produced from *Nerium oleander* showed an efficient antibacterial activity against *E.coli* and *S.aureus* with zone of inhibition 10mm and 21mm respectively (27).

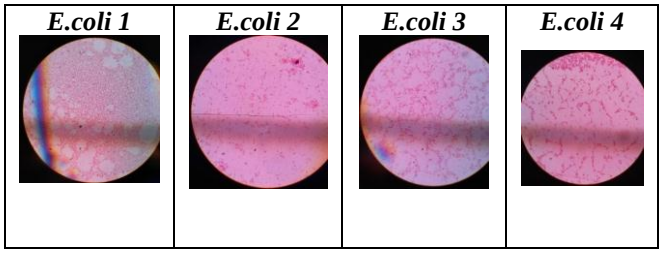

Figure.1: Microscopic images of *E.coli* strains.

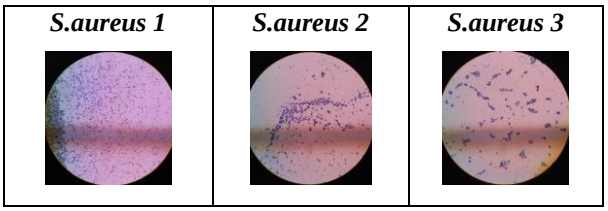

Figure.2: Microscopic images of *S.aureus* strains.

	Indole	Methyl red	VogeProskauer test	Citrate test
E.c1	+	+	-	-
E.c 2	+	+	-	-
E.c 3	+	+	-	-
E.c 4	+	+	-	-
S.a 1	-	+	-	-
S.a 2	-	+	-	-
S.a 3	-	+	-	-

Table.1: Indicating the results of IMVIC tests.

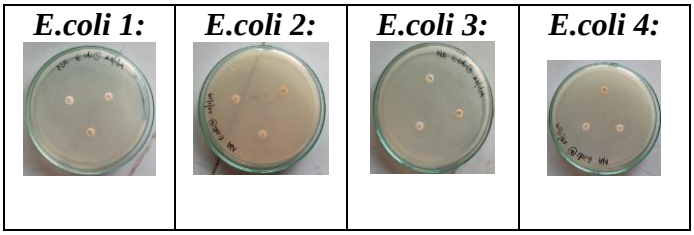

Figure 3: Indicating the antibiotic sensitivity of all the strains of *E.coli*.

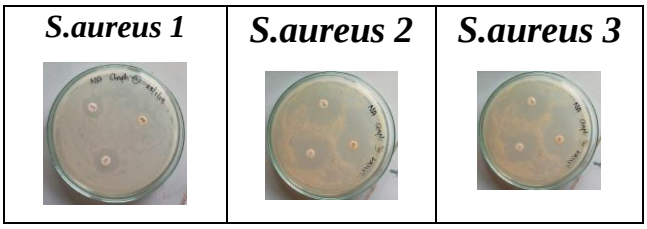

Figure 4: Indicating the antibiotic sensitivity of all the strains of *S.aureus*.

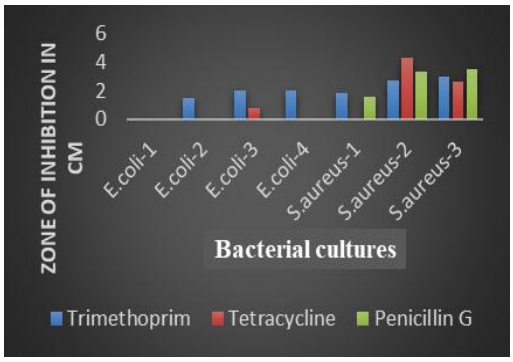

Figure 5: Indicating the results of antibiotic sensitivity assay

Figure 6: Indicating the antibacterial activity of the plant extract.

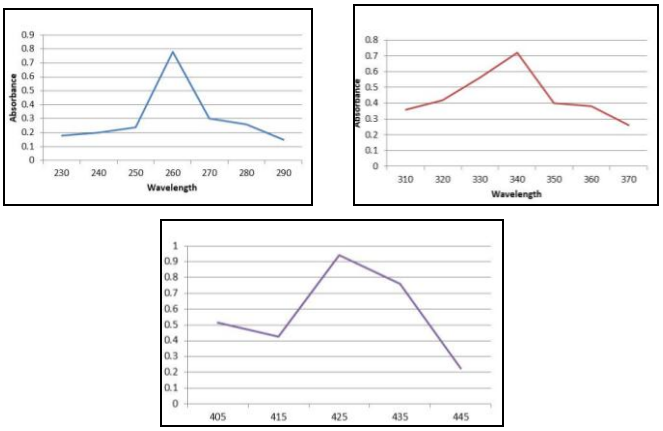

Figure 7: UV-Vis absorption spectra of FeNPs, CuNPs and AgNPs at 1mM concentration of AgNO3, FeCl3 and CuSO4

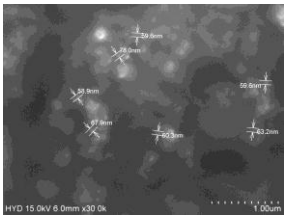

Figure 8: Indicating SEM image of FeNPs.

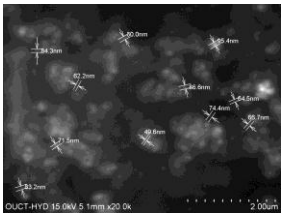

Figure 9: Indicating SEM image of CuNPs.

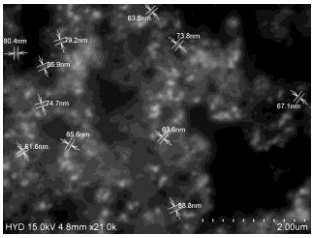

Figure 10: Indicating SEM image of AgNPs.

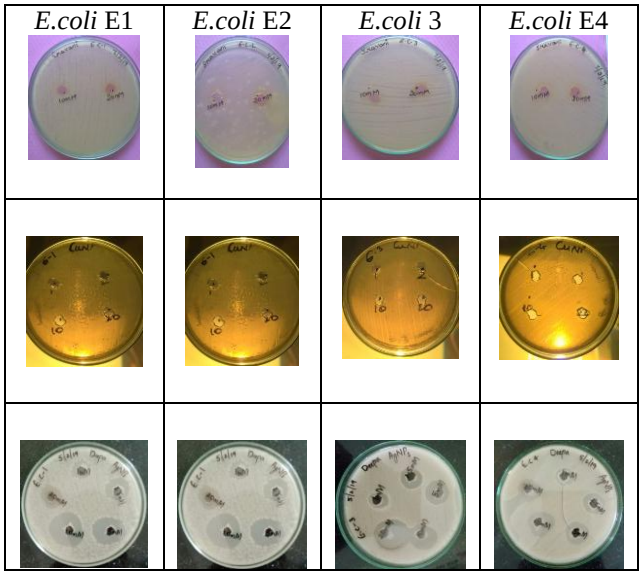

Figure 11: Indicating the antibacterial activity of FeNPs, CuNPs and AgNPs against *E.coli* (E1, E2, E3 and E4)

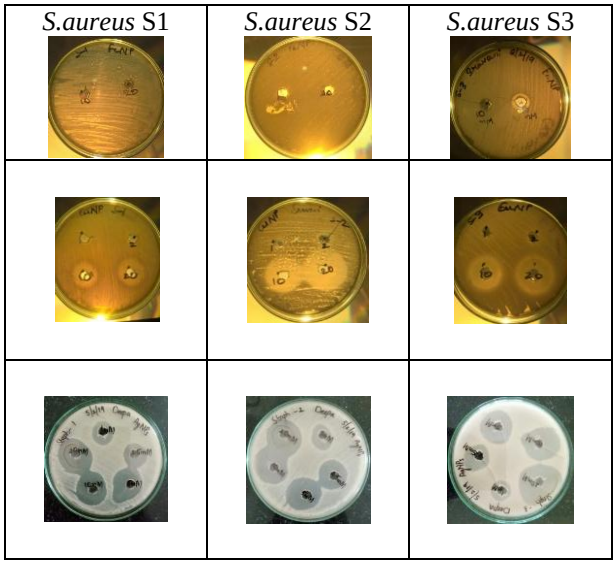

Figure 12: Indicating the antibacterial activity of FeNPs, CuNps and AgNPs against *S.aureus* (S1, S2 and S3) strains.

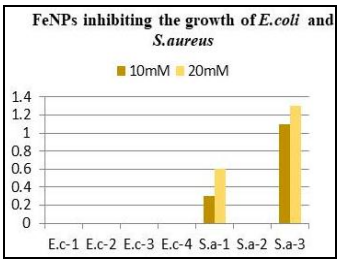

Figure 13: Indicating the zone of inhibition by FeNPs

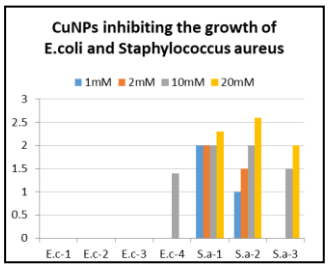

Figure 14: Indicating the zone of inhibition by CuNPs

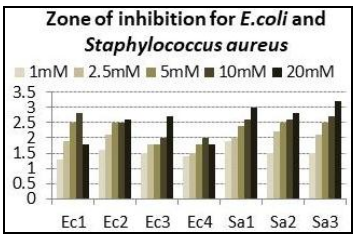

Figure 15: Indicating the zone of inhibition by AgNPs

IV. Conclusion and Future Scope

In the present study, green synthesis of Iron, Copper and Silver nanoparticles using the leaf extract of Neem (*Azadirachta indica*) was reported. The UV-Vis analysis showed the absorption values of Iron, Copper and Silver nanoparticles at 260nm, 340nm and 425nm respectively. The SEM analysis revealed the size of Iron, Copper and Silver nanoparticles as 58.9-78.0nm, 49.6-95.4nm and 63.6-88.8nm respectively. When the antibacterial studies were done, Silver nanoparticles showed the maximum zone of inhibition against the obtained animal pathogens *E.coli I* (E1, E2, E3 and E4) and *S.aureus* (S1, S2 and S3) when compared to Iron and Copper nanoparticles. Such studies not only solve the problem of antibiotic resistance but also evaluate the synthesis of nanoparticles using natural sources.

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