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RESEARCH ARTICLE

GREEN SYNTHESIS OF SILVER NANO PARTICLES USING LEAF EXTRACT OF *COCCINIA INDICA* AND ITS ACTIVITY AGAINST MULTIPLE DRUG RESISTANT (MDR) STRAINS OF BACTERIA

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ABSTRACT

This study evaluate antibacterial property of green synthesized silver nanoparticles *Coccinia indica* extract as reducing agent against multiple drug resistant bacteria (MDR) such as *E. coli, S. aureus* and *Pseudomonas aeruginosa.* Silver nanoparticles were synthesized through *Coccinia indica* extract and characterized using UV-VIS spectroscopy, SEM, TEM techniques. The antibacterial activity assays were done against all bacterial pathogens by well diffusion method. The diameter of silver nanoparticles was predominantly found within the range 100-300 nm. The absorption peak at 420 nm broadens with increase in time indicating polydispersity nature of the nanoparticles. The present study showed that *P. aeruginosa* and *E.coli* was found to be resistant against Ampicillin, Cephotaxime, Ceftazidime, Clotrimazole and Gentamycin. *S. aureus* was resistant toward Methicillin, Vancomycin, Imipenem and Tobramycin. In this study, the Green SNPs showed excellent zone of inhibition against Pseudomonas aeruginosa (22 mm) whereas least zone of inhibition was found against S. aureus (8 mm). It has been found that nanoparticles treated with herbal solutions at different concentrations resulted into inhibition of *S. aureus* at 12.5 µg/disc and slightly increased concentration of SNPs i.e. 1.56 µg/ml is the minimum concentration found inhibitory to the growth of MDR strains of *Pseudomonas* whereas SNPs didn't show any zone of inhibition against *E. coli*. The present findings suggest that green synthesis of nanoparticles are simple, quite effective method that can be used as an alternative antibacterial agent against diseases caused by multiple drug resistant pathogens.

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INTRODUCTION

In the present era, pharmaceutical and biomedical sectors are facing the challenges of continuous increase in the multidrug-resistant human pathogenic microbes. Re-emergence of MDR microbes is facilitated by drug and / or antibiotic resistance, which is acquired way of microbes for their survival and multiplication in uncomfortable environments. MDR bacterial infections lead to significant increase in mortality, morbidity and cost of prolonged treatments (Rai, 2012). Bacteria acquire resistance to antibiotics due to enzymatic deactivation of antibiotics, decreased cell wall permeability to antibiotics, altered target sites of antibiotics, efflux mechanism to remove antibiotics and may be due to increased mutation rate as a stress response (Magiorakos, 2011). Once an individual is infected with MDR bacteria, it is not possible to cure easily and the patient has to spend more time in the hospital and requires a multiple treatment of broad spectrum antibiotics, which are less effective, more toxic and more expensive. Therefore, development of or modification in antimicrobial compounds to improve bactericidal potential is a priority area of research in this modern time (Rai, 2012). The microbial infections are commonly treated with the antibiotics, drugs and many other potent antibacterial agents. Multi drug resistance (MDR) is a condition that enables disease causing micro-organisms to resist distinct antimicrobials, antibiotics, antifungal drugs, antiviral medications, anti parasitic drugs and chemicals of wide variety of structure and functions targeted at eradicating the organism. Recognizing different degrees of MDR, the terms extensively drug resistant (XDR) and pandrug resistant (PDR) have been introduced (Khan Rosina, 2009). MDR, XDR or PDR, for all the three definitions, non-susceptibility refers to either a resistant, intermediate or non-susceptible result obtained from in-vitro antimicrobial susceptibility testing. A group of international experts came together through a joint initiative by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), to create a standardized international terminology with which to describe acquired resistance profiles in *Staphylococcus aureus,*

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Enterococcus spp., Enterobacteriaceae (other than *Salmonella and Shigella*), *Pseudomonas aeruginosa* and *Acinetobacter spp.*, all bacteria often responsible for healthcare-associated infections and prone to multidrug resistance. Emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for infections caused by these bacteria.

For thousands of years, natural products have been used in traditional medicine all over the world and predate the introduction of antibiotics and other drugs. The antimicrobial efficacy attributed to some plants in treating diseases has been great (Hiremath Jyothi, 2014). Presently in an era where we need to improve this antimicrobial efficacy to greater levels so as to overcome superbugs infections. In the last decade, herbal drug development has good acceleration in pharma industry due to the side effects and massive use of chemicals in allopathy. By all possible ways scientists are trying to get off from this resistance problem (Chaudhari, 2012). In the 21st century, nanotechnology is emerging as cutting edge technology and has incredible applications in physics, chemistry, biology, material science and medicine. The major thrust has been developing new materials and examining their properties by tuning the particle size, shape and distribution. Metal nanoparticles have been extensively studied due to their specific characteristics such as catalytic activity, optical properties, electronic properties, antimicrobial properties and magnetic properties (Dubey Manish, 2012). Currently, researchers are focusing on the synthesis of nanoparticles using micro-organisms and plant extracts (Kagithoju Srikanth, 2014). Conventional methods are energy intensive, employ toxic chemicals which are expensive and inefficient. Therefore, there is a growing need for the use of bio-compatible, non-toxic, cost effective and eco-friendly methods for production of metal nanoparticles (Das J, 2013).. The antibacterial effects of silver salts have been acknowledged since years and today silver is used to control bacterial growth in a variety of applications including dental work, catheters and burn wounds and its spectrum is rather broad (Arunachalama Rajeswari, 2012).

Coccinia indica, the *ivy gourd*, also known as baby watermelon, little gourd, tindora or gherkin is a topical vine. It is also known as *Cephalandra indica, Kovakka* and *Coccinia grandis*.

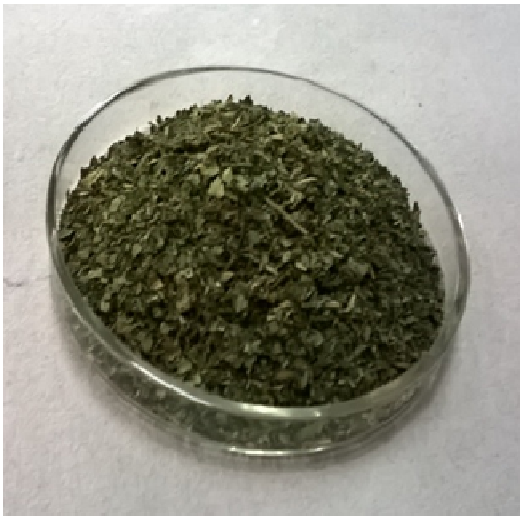


Figure 1. *C. indica* dried leaves

Coccinia indica commonly known as '*Ivy gourd*' and *'Kundru*' in hindi is a perennial tendril climber, available in wild and cultivated form. It is the native of Central Africa and Asia. It is well distributed naturally in China, topical Asia, Australia, the Phillippines, Indinesia, Thailand, Malaysia and Myanmar (Singh, 2014). It is considered as a valuable vegetable by the indigenous people of Southeast Asia and India. Every part of this plant is valuable in medicine for ringworm, psoriasis, small pox and other itchy skin eruption and ulcers. In traditional medicine, fruits have been used to treat leprosy, fever asthma, bronchitis and jaundice. The fruit possesses mast cell stabilizing, anti anaphylactic and antihistaminic potential. Roots are used to treat osteoarthritis. A paste made of leaves is applied to skin to treat scabies. *C. indica* has anti diabetic, hypoglycemic, anti-inflammatory, analgesic, hepato protective, antioxidant and anti mutagenic activity. Researchers have revealed that the eight different solvent extracts of *C. indica* fruit were tested against six gram negative and gram positive bacteria. The crude extracts of *C. indica* exhibited moderate to significant antibacterial activity against all tested bacteria (Rao M. Linga, 2009). Inspired green synthesis of silver nano particles is evolving as an important branch of nanotechnology. Traditionally these are manufactured by wet chemical methods which require toxic and flammable chemicals Shaheen Syed Zeenat, (2009). The present study reports an economic and eco-friendly green synthesis of silver nano particles using *Coccinia indica* aqueous leaf extract from 1mM and 3mM silver nitrate solution and the effect of the synthesized Ag nano particles on MDR strains of bacteria.

Methodology

1.Preparation of leaf extract

Coccinia indica leaves were collected and washed several times with distilled water before used for extraction. A 20 gm of this plant leaves were dried to remove moisture and stirred with 100 ml sterile distilled water and kept in water bath at 80°C for 3 min. The extract was filtered through a nylon mesh (0.2 µm) followed by Whatmann filter paper 1. The filtrate was used as a reducing agent and stabilizer. The extract can be stored in the refrigerator for further use (Wasnik Durgesh D, 2014).

2.Synthesis of Silver nanoparticles

For synthesis of SNPs, 5 ml of leaf extract was added to 45 ml of 1mM and 3mM aqueous silver nitrate solution in 250 ml conical flask. The flask was then incubated in dark at room temperature. A control set up was also maintained without leaf extract, The SNPs solution thus obtained was observed for color change. It was then purified by repeated centrifugation at 10,000 rpm for 15 min followed by re-dispersion of the pellet in double distilled water. Then the SNPs were stored at 4°C for further use (Singh, 2014).

3.Characterization studies

The biosynthesis of SNPs was monitored periodically by scanning the aliquot sample in a wavelength range of 200-1100 nm and recording the absorption maxima in UV Visible spectrophotometer at a resolution of 1nm (Singh,2014). The nanoparticles produced were primarily characterized by UV-Visible spectrophotometer. SEM analysis was carried out to understand the topology of SNPs. For scanning electron

microscope, the sample was prepared with a drop of colloidal solution of nanosilver on a carbon-coated copper grid and a setting completely dried by vacuum desiccators (Fig. 1). The maxima of the UV-Visible spectra of the solution occurred at 420 nm and the following image was obtained in the SEM analysis (see Graphs).

4.Antibiotic susceptibility testing of the bacterial strains

The bacterial strains *E. coli, S. aureus,* and *Pseudomonas aeruginosa* were used for study and were procured from Medical laboratory of Post Graduate Department of Microbiology, R. T. M. Nagpur University, Nagpur. These bacterial strains were checked for their antibiotic susceptibility pattern. For the Gram negative bacteria, Levofloxacin, Cefotaxime, Ampicillin, Ofloxacin, Tobramycin, Clotrimazole, Ceftazidime and Gentamycin were used. For gram positive bacteria, Methicillin, Vancomycin, Daptomycin, Linezolid, Imipenem, Cefoxitin and Cephotaxime were used.

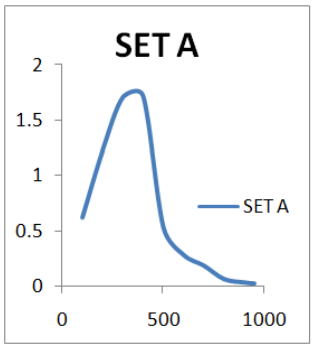
5.Determination of MIC of synthesized SNPs

The bactericidal studies were done by agar well diffusion method (Perez *et al*., 1990) (Shaheen Syed Zeenat *et al*., 2009). The bacterial strains were inoculated into nutrient broth which was then incubated at 37°C for 6 – 8 hours. This culture was used for making lawn culture on the plate containing sterile 20 ml Muller Hinton Agar. MIC of SNPs for MDR strains were determined by diluting the standard 100µg/ml SNPs solution to 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml,1.562 µg/ml. In this way, each dilution (50µl) was introduced into 6 mm wells in the agar plates already seeded with six hour incubated broth culture of organisms. All plates were incubated aerobically at 37°C for 24 hours and then were observed for the zone of inhibition.

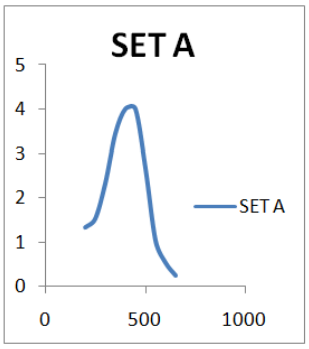
RESULTS AND DISCUSSION

The present study shows that *P. aeruginosa* and *E.coli* was found to be resistant against Ampicillin, Cephotaxime, Ceftazidime, Clotrimazole and Gentamycin. *S. aureus* was resistant toward Methicillin, Vancomycin, Imipenem and Tobramycin. (Table 2) In this study, the Green SNPs showed excellent zone of inhibition against *Pseudomonas aeruginosa* (22 mm) whereas least zone of inhibition was found against *S. aureus* (8 mm) (Table 1). It has been found that nanoparticles treated with herbal solutions at different concentrations resulted into inhibition of *S. aureus* at 12.5 µg/disc. This study shows that slightly increased concentration of SNPs i.e. 1.56 µg/ml is the minimum concentration found inhibitory to the growth of MDR strains of *Pseudomonas* and SNPs didn't show any zone of inhibition against *E. coli* (Table 3). Kagithoju *et al.* used 50 µl of herbal nanoparticles solution in combination with herbal extract. The SNPs in combination with herbal extracts of *Carica papaya* is found to be inhibitory for *P. aeruginosa* (Kagithoju Srikanth, 2014). Whereas SNPs alone at the concentration 50µg/ml are found to be extensively effective against these infectious bacteria. It has also been studied that synergistic effect of antibiotics and SNPs has enhanced the zone of inhibition of disease causing bacteria (Chaudhari, 2012). In the present study most of the antibiotics used against *P. aeruginosa* were found to be in effective against it and hence silver nano particles are successfully used against these MDR strains and are found more effective though in slightly increased concentration than the antibiotics. Studies conducted by Khushbu Singh *et al*. shows that the MIC for *P. aeruginosa* isolated from burn samples was found to be 6.25µg/ml (Singh, 2014). The highest zone size was found to be 21mm while in the present study it was found to be 22mm. The results in the present study correlate with the studies

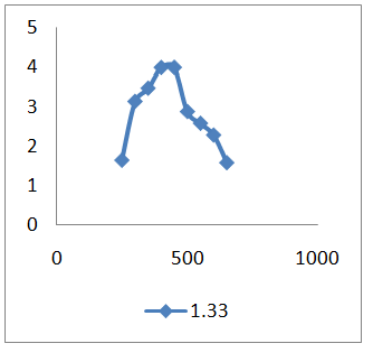
A. Characterization studies UV-Visible Spectrophotometer (Absorption Spectra)



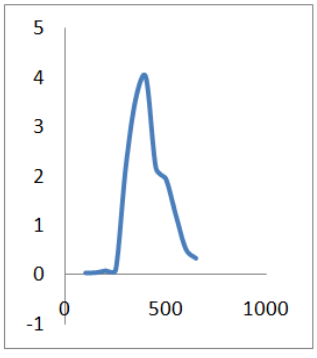
Graph 1: Set A (Zero time)For1mM $AgNO_3$



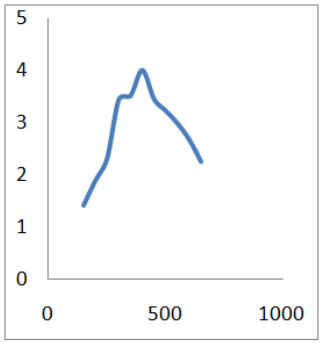
Graph 2: Set A after 24 hours



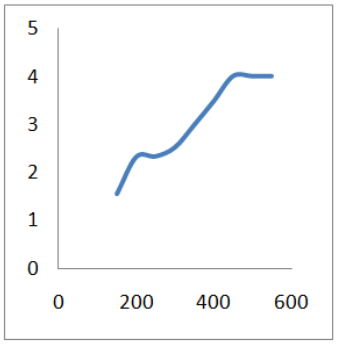
Graph 3: Set A (after 48 hours)



Graph 4: Set B (Zero time) for 3mM $AgNO_3$



Graph 5: Set B (after 24 hours)



Graph 6: Set B (after 48 hours)

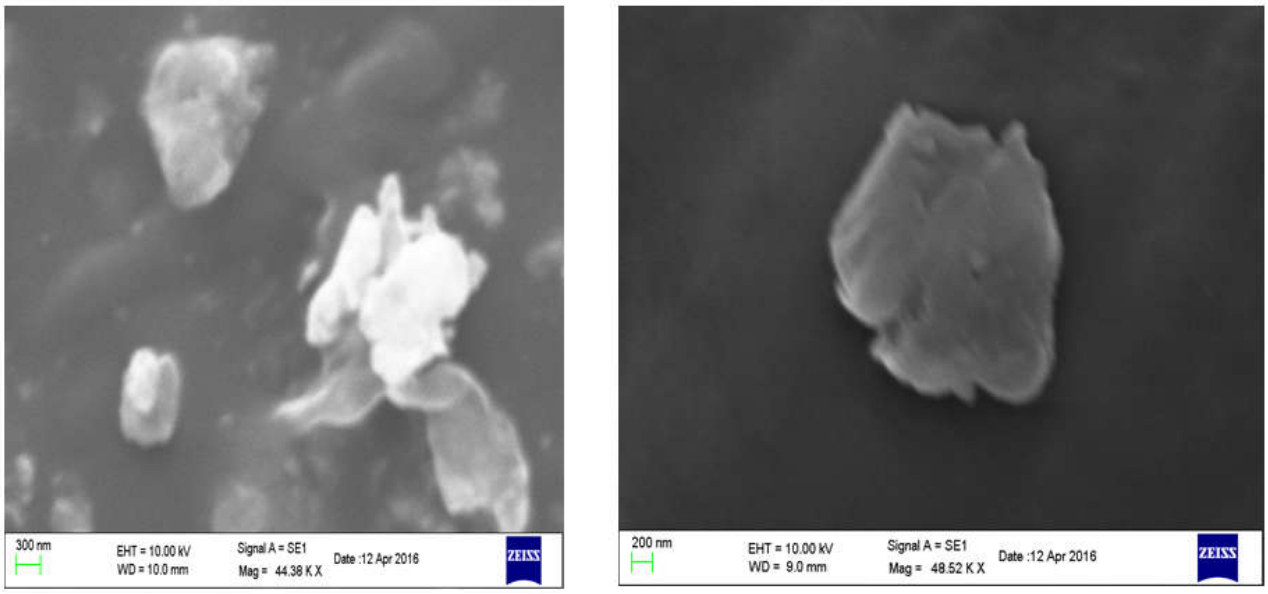


Fig.2. SEM of Green Silver Nanoparticles

Table 1. Antibacterial action of SNPs against MDR strains of bacteria

S.No.	MDR bacterial strains	Zone of Inhibition (in mm)	
		1mM	3mM
1.	*E. coli*	Nil	Nil
2.	*P. aeruginosa*	18mm	22mm
3.	*S. aureus*	6mm	8mm

Table 2. Antibiotic susceptibility test for clinical isolates

S. No	Antibiotics used	Zone of inhibition in mm		
		S. aureus	*E.coli*	*P. aeruginosa*
1	Tobramycin	16 (R)	20 (S)	18 (S)
2	Ofloxacin	20 (S)	18 (S)	20 (S)
3	Levofloxacin	18 (S)	22 (S)	21 (S)
4	Ampicillin	---	No zone	No zone
5	Cephotaxime	---	10 (R)	12 (R)
6	Ceftazidime	20 (S)	18 (S)	No zone
7	Clotrimazole	---	No zone	No zone
8	Gentamycin	12 (R)	No zone	No zone
9	Methicillin	No zone	---	---
10	Vancomycin	No zone	---	---
11	Daptomycin	22 (S)	---	---
12	Linezolid	18 (S)	---	----
13	Imipenem	14 (R)	---	28 (S)
14	Cefoxitin	20 (S)	---	---

S – Sensitive R – Resistant

Table 3. Determination of MIC of SNPs for *P. aeruginosa*

Sr. No.		1	2	3	4	5	6
Concentrations of SNPs (µg/ml)		50	25	12.5	6.25	3.125	1.562
Zone of inhibition (mm)	*Ps. aeruginosa*	18	16	13	11	10	---
	S. aureus	5	6	6	---	---	---

conducted by Singh *et al*. where the lowest values of MIC were recorded as 6.25 µg/ml nd the highest recorded was 200µg/ml. Durairaj *et al.* studied the antibacterial activity of purchased AgNPs (size 20-30nm) against 10 isolates of *P. aeruginosa* comprising of 5 MDR strains with an inhibition zone of 11 mm observed with10 µg dose of the nanoparticles. The nanoparticles exhibited MIC of 50 µg/ml when added at the lag phase and the sub inhibitory concentration was measured as 100 µg/ml. In presently studied experiment, when we compared the antibacterial activity of AgNPs and plant extract, it was found that silver AgNPs have shown more antibacterial activity than plant extracts.

Wavelength on X-axis and Absorbance on Y-axis for all the above graphs. The absorption maxima was obtained at 420nm which clearly indicates the synthesis of Silver nanoparticles in the solution. Further SEM measurements confirmed the synthesis of SNPs in the solution. The present results clearly shows that the conventional plant extract showing some antibacterial activity but not much activity as AgNPs does against these MDR bacterial strains. It clearly indicates that these green AgNPs have shown considerable amount of activity than that of plant extract. Antibacterial activity of AgNPs significantly increases by more than 12-15% at very lower concentration than that of plant extract. The results

showed that SNPs synthesized from *Coccinia indica* leaf extract possess discrete antibacterial activity at different concentrations of 50µg/ml – 1.58µg/ml. The zone of inhibition ranges from 8mm ± 0.5 to 18 mm ± 0.5. The standard 1mg/ml solution of SNPs showed maximum zone of 18 mm and 22 mm for 1mM and 3mM $AgNO_3$ solution against MDR *Pseudomonas aeruginosa* strains. Moreover, when antibacterial activity of AgNPs synthesized from that plant extract was compared with the plant extract alone, it clearly shows that the conventional plant extract showing not much activity as AgNPs does against these MDR bacterial strains, even taken in amount10 times more than AgNPs. It clearly indicates that these green AgNPs have shown considerable amount of activity than that of plant extract. Antibacterial activity of AgNPs significantly increases by more than 12-15% i.e. zone of inhibition comes at concentration of mg/ml in case of plant extract and µg/ml in case of AgNPs (Singh, 2014).

Conclusion

The present study comes to the conclusion that even at very small concentration (in µg/ml) SNPs from leaf extract of *Coccinia indica* possess very good antibacterial activity which makes them a potent source of antibacterial agent MDR strains of *Pseudomonas aeruginosa*. Also green synthesis can potentially eliminate the problem of chemical agents that may have adverse effects, thus making nanoparticles more compatible with the eco-friendly approach. Hence, the results from this study are promising and prove to be an important step in the direction of minimizing the risk of superbugs infections by *P. aeruginosa*

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