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Biosynthesis, Characterization and Study of Antimicrobial Activity of Copper and Silver Nanoparticles

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ABSTRACT: *Nanotechnology is gaining a lot of importance in various fields especially in medical field as many organisms are becoming resistant. Emerging infectious diseases and the increase in incidence of drug resistance among pathogenic bacteria have made the search for new antimicrobials inevitable. In the current situation, one of the most promising and novel therapeutic agents are the nanoparticles. Hence these nanoparticles can be used as antimicrobial agents to overcome this problem. In this study, copper and silver nanoparticles were made using a plant source and a microbial source. The synthesized nanoparticles were characterized using UV-Vis spectroscopy and FTIR. Also antimicrobial activity of these synthesized nanoparticles was checked for clinical isolates (fresh wound isolates and antibiotic resistant organisms) and MIC was carried out.*

Keywords: *Nanoparticles, antimicrobial activity, synthesis, silver, copper, characterization*

I. Introduction

Nanotechnology is science, engineering, and technology conducted at the nanoscale. Nanoscience and nanotechnology are the study and application of extremely small things and can be used across all the other science fields, such as chemistry, biology, physics, materials science, and engineering. The advance in nanotechnology has enabled to utilize particles in the size of the nanoscale. The use of noble metals at nanosizes to treat many conditions is gaining importance. The recent development in nanotechnology has provided tremendous impetus in this direction due to its capacity of modulating metals into nanosizes and various shapes, which drastically changes the chemical, physical and optical properties and their use. Nanobiotechnology is considered to be the unique fusion of biotechnology and nanotechnology by which classical micro-technology can be merged to a molecular biological approach in real. Through this methodology, atomic or molecular grade machines can be made by mimicking or incorporating biological systems, or by building tiny tools to study or modulate diverse properties of a biological system on molecular basis. Nanobiotechnology may, therefore, ease many avenues of life sciences by integrating cutting-edge applications of information technology & nanotechnology into contemporary biological issues. Nanoparticles (NPs) are particles between 1 and 100 nano-meters in size. In nanotechnology, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential applications in biomedical, optical and electronic fields. Nanoparticles are of great scientific interest as they are, in effect, a bridge between bulk materials and atomic or molecular structures. Nanoscience and nanotechnology have found their way into the fields of biotechnology and medicine.

Copper is a chemical element with the symbol Cu (from Latin: cuprum) and atomic number 29. Copper is well known for its antimicrobial activity and finds application in wound healing, skin re-modulation and anti-inflammatory therapies. Antimicrobial activity of copper helps to reduce the microbial load at the site of wound and enhances the pace of healing. Surfaces of copper nanoparticles affect / interact directly with the bacterial outer membrane, causing the membrane to rupture and killing bacteria.

Silver is a chemical element with the symbol Ag and atomic number 47. Dilute silver nitrate solutions and other silver compounds are used as disinfectants and microbiocides (oligodynamic effect), added to bandages and wound-dressings, catheters and other medical instruments. The silver ion (Ag+) is bioactive and in sufficient concentration readily kills bacteria in vitro. Silver and silver nanoparticles are used as an antimicrobial in a variety of industrial, healthcare and domestic applications.

Ocimum tenuiflorum, also known as *Ocimum sanctum*, holy basil, or tulsi, is an aromatic plant in the family Lamiaceae which is native to the Indian subcontinent. *O. sanctum* is well known for its medical use. *O. sanctum* extracts have some antibacterial activity against *E. coli, S. aureus* and *P. aeruginosa*.

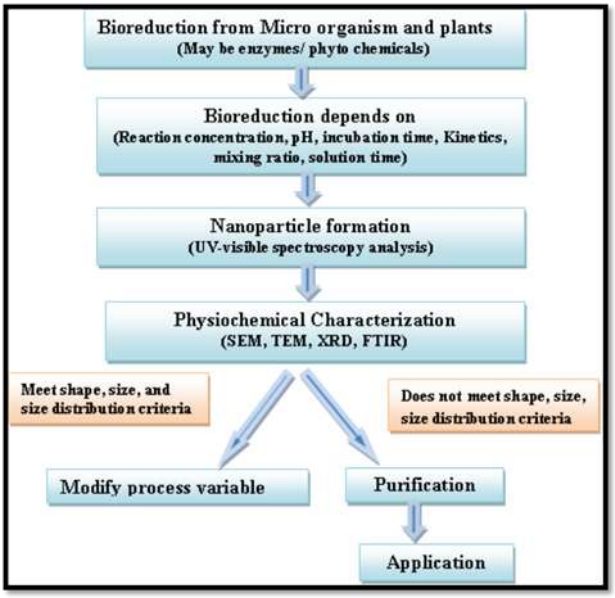


Fig. 1- General outline of synthesis of NPs

Metal nanoparticles have microbiocidal action and can reduce specific harmful bacteria linked to potentially deadly microbial infections.

II. Methods And Materials

1) Biosynthesis of Cu NPs by microbial method

(a) Culture used: The laboratory cultures of *Pseudomonas aeruginosa* were used. The culture was first streaked on Nutrient agar slant to check for pigmentation and gram staining was carried out to check the grams nature of the culture. *P. aeruginosa* is gram negative bacilli.

(b)Purification of the culture: The culture was then isolated on selective plate to confirm for *P. aeruginosa*. The selective plate used was Cetrimide agar. Cetrimide agar is a type of agar used for the selective isolation of the gram-negative bacterium, *P. aeruginosa*. As the name suggests, it contains cetrimide, which is the selective agent against alternate microbial flora. Cetrimide also enhances the production of *Pseudomonas* pigments such as pyocyanin which show a characteristic blue-green colour.

(c) Preparation of cell free supernatant: *P. aeruginosa* was first grown in Nutrient broth for 3-4 days to get a luxuriant growth. The broth was incubated at 37^0C. After the incubation time the culture was then ultra-centrifuged at 14,000 rpm for 15 minutes to remove all the cell debris. The supernatant was the passed aseptically through 0.4µm membrane filter (Millipore filter) to get a cell free extract of *P. aeruginosa*. This .cell free extract can be further used for synthesis of NPs.

Fig. 2 - Membrane filter (Millipore filter) to obtain a cell free extract

(d) Selection of optimum conditions for biosynthesis of NPs: Biosynthesis of Cu NPs depends on various factors like pH, temperature, rpm and concentration of the copper ion. Cell-free supernatant of culture was exposed to different pH- 5.0, 6.5 7.0 and 9.0, temperatures- 27, 37 and 45 °C and 100 rpm and static conditions by using 1 mM CuSO4 concentration to select optimum conditions for the synthesis of CNPs. CuSO4 concentration 1 mM was used at normal conditions pH (7.0). Change in the colour of reaction mixtures and surface plasmon resonance (SPR) of synthesising nanoparticles were considered for selecting the optimum condition. Change in colour was examined visually and change in SPR was determined spectrophotometrically. The desired change in colour is blue to green.
(e) Synthesis of Cu NPs: *P. aeruginosa* was cultured in NB broth. The flask was harvested to get the cell-free supernatant of culture after maximum growth. After adjusting the pH of cell-free supernatant of culture at 7, equal volume of 4 mM CuSO4 solution was added. The same procedure was carried out by using same pH of cell free supernatant of P. aeruginosa but with different concentrations of CuSO4 – 5mM, 7mM, 8mM and 10mM solutions. The flask was kept in orbital shaker at 27 °C and 100 rpm.

2) Biosynthesis of Cu and Ag NPs by Phytochemical method
(a) Sample used: *Ocimum sanctum*, a traditional medicinal plant of India also known as Tulsi have been used as a source of bio-reduction and stabilizers for synthesis of Cu and Ag NPs and the constituents such as alkaloids, glycosides, tannins, saponins and aromatic compounds may be responsible for the synthesis of nanoparticle.
(b) Preparation of phytochemical extract of Tulsi: The plant of interest was collected from a garden. The leaves were washed & cleaned thoroughly with tap water and distilled water to remove debris. The first method used was using fresh leaves to make the phytochemical extract. 2.5 gm leaves which are chopped finely are used. These leaves are added to 50 ml of st distilled water in a flask and were boiled for 10 mins. This mixture was then filtered through a filter paper. This phytochemical extract was then used for further synthesis of NPs. The second method used was the use of oven dried leaves which were dried for a day and then powdered using domestic blender. The plant broth preparation was made by a 10gm of the dried powder boiled for 10 minutes with 100 ml of distilled water. The resulted infusion is filtered and used as a reducing agent and stabilizer.
(c) Synthesis of NPs:
The extract (1ml) is mixed with 100ml of AgNO3 and CuSO4 solutions (100 ml each, 1mM). This mixture is incubated for 2 days at RT/ static conditions until the desired colour change is observed.

3) Characterization of the metal NPs:
The metal NPs (Ag and Cu) thus formed are then characterized by UV-Vis spectroscopy and FTIR.

4) Confirming the clinical isolates by plating on Selective medium

The clinical isolates (wound organisms and antibiotic resistant organisms) which were provided by KEM hospital were confirmed by isolating the isolates on selective plates. The isolates provided by KEM hospital were pure isolates which were grown on selective plates and then subcultured on NA slant. The isolates received were *S. aureus, E. coli, P. aeruginosa, V. cholerae, K. pneumoniae, Proteus species, E. faecalis. S. aureus* was streaked on Baird Parker agar. *E. coli* and *K. pneumoniae* were isolated on MacConkey' s agar plate. *P. aeruginosa* was isolated on Cetrimide agar. *V. cholerae* was isolated on Thiosulfate-citrate-bile salts-sucrose agar (TCBS). *Proteus sp* was isolated on Cystine lactose electrolyte deficient agar (CLED). *E. faecalis* was isolated on Enterococcus agar. The plates were incubated at 370C for 24- 48 hrs and were observed for growth.

5) Antibiogram of the clinical isolates:

The clinical isolates were swabbed on the Mueller Hinton agar plates. Then for each isolate swabbed on the plate respective antibiotics were placed on the plate. The antibiotics used were Gentamicin, Chloramphenicol, Tetracycline, Amoxicillin, Methicillin, Vancomycin, Kanamycin, Streptomycin, Ampicillin, Piperacillin, Cefixime, Amikacin, Trimethoprim and Levofloxacin (concentrations as mentioned in the table). These antibiotics used were of Himedia. The isolates used were *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Methicillin Resistant Staphylococcus aureus, Klebsiella pneumoniae, Vibrio cholerae, Proteus species, Enterococcus faecalis*. These cultures were adjusted to 0.1 O.D. This is done so that the number of cells remains same. Then antimicrobial activity of Cu and Ag NPs was checked against the above mentioned cultures. For that first the NP solution was spun to 14,000 rpm for 15 minutes and then the pellet settled at the bottom was dried and mixed with st distilled water. This then was used to check antimicrobial activity by adding this mixture in the wells on Muller Hinton agar. These plates were then incubated at 370C for 1-2 days.

6) Minimum Inhibitory Concentration:

To perform MIC, agar dilution method is used. Mueller Hinton agar is used in this method. The sample that is NPs are used by first spinning the colloidal solution at 14,000 rpm for 15 mins and then mixing the Particles which settled down in st distilled water. This is then further diluted (double dilution) upto 1:8 dilution. This mixture (1 ml) is then added to molten agar butts (19 ml) and then mixed and poured onto a st petriplate. The isolates are O.D adjusted. Further, the isolates are spot inoculated one by one (5µl) by a micropipette on the solidified plate and then the plate is incubated at 37^{o}C for 24-48 hours. The results can be interpreted by inhibition of the isolate for a particular dilution.

III. Results

1) Biosynthesis of Cu NPs by microbial method

(a) Culture used: The culture was grown on Nutrient agar slant and gave a bluish green growth on the slant after incubation. Gram staining was carried out and was found to be gram negative, coccobacilli.

(b) Purification of culture: The culture was then streaked on Cetrimide agar plate and after incubation for 2 days purple growth appeared which was confirmed that the culture was P. aeruginosa.

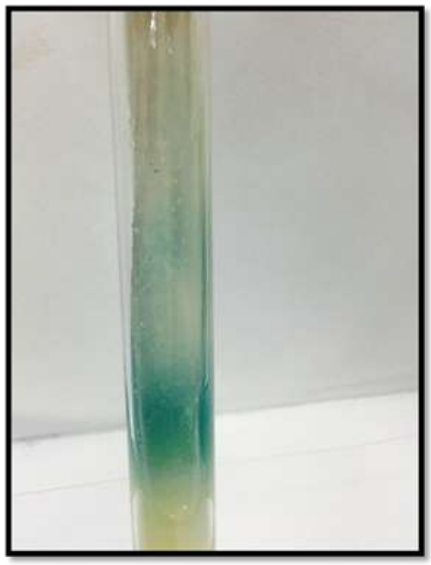


Fig. 3 P. aeruginosa grown on NA slant

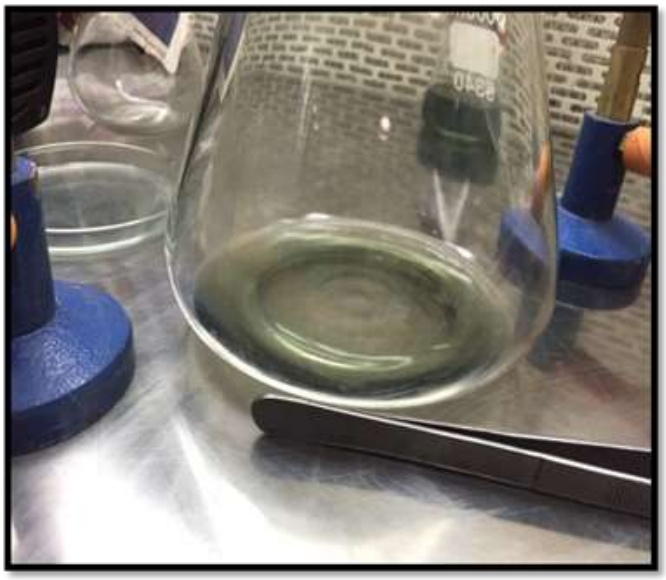


Fig.4 cell free supernatant collected in the flask

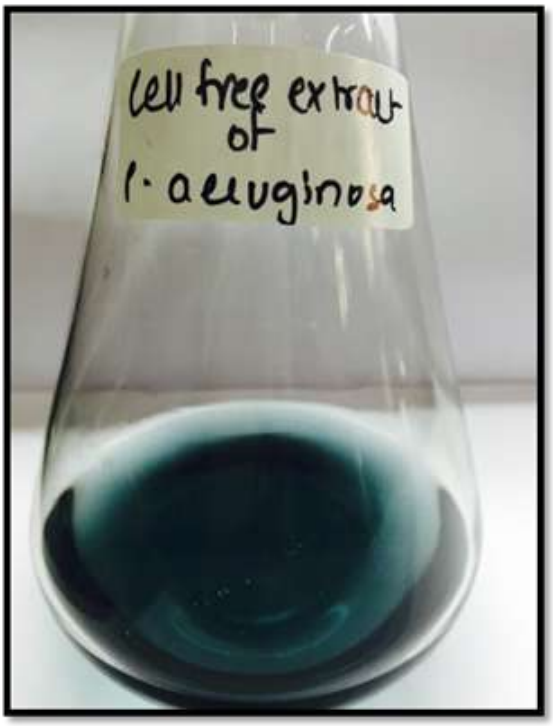
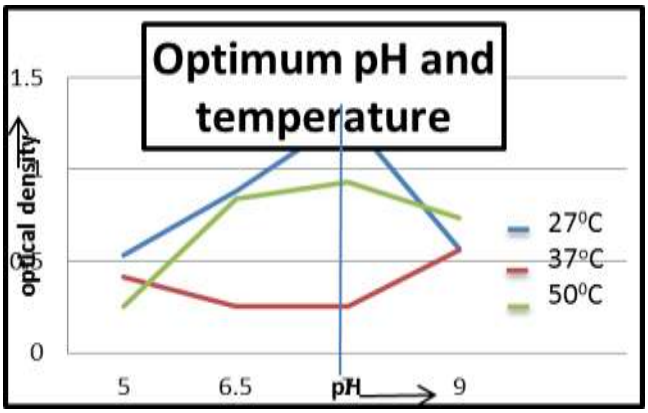


Fig. 5 cell free extract of P. aeruginosa

Table 1 Selection of λ max for optimization of NPs

Wavelength(nm)	O.D
300	4
305	4
310	4
315	3.94
320	3.639
325	2.884
330	2.450
335	2.083

The λmax was found to be 310nm. The optimization was carried out at this wavelength.



Graph 1- Optimization of Cu NPs (pH and temperature)

The optimum temperature for the synthesis of Cu NPs was found to be 270C and the optimum pH was found to be 7. The pH of the cell free extract was 7. The optimum rpm was 100.
The formation of Cu NPs is indicated by the colour change that is from blue colour, which forms after the addition of CuSO4 (pH 7) to the cell free extract (pH 7) aseptically in equal volumes, to green colour.

Fig.6 cell free extract+ $CuSO_4$ (initial- blue in colur)

Fig. 5.3 (b) cell free extract + CuSO4 (after incubation- turning green in colour)

2 Biosynthesis of Cu and Ag NPs by Phytochemical method

(a) Preparation of phytochemical extract of Tulsi: The extract was made using fresh tulsi leaves which were finely chopped and using dried leaves which were dried and then powdered and was added to st distilled water and boiled. Both the extracts turn brown in colour after heating. The boiled extract is filtered using Wharmann No 1 filter paper. The filtrate collected was further used for synthesis of Ag and Cu NPs.

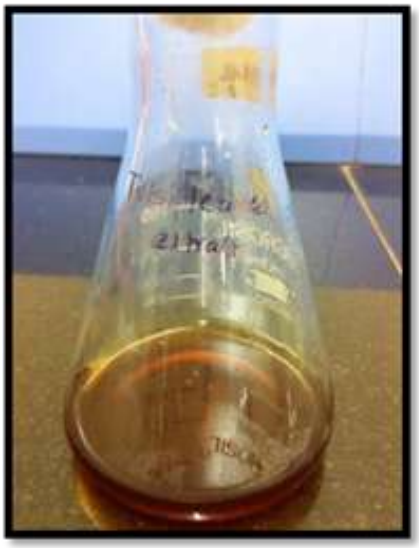


Fig. 7 extract of *Ocimum sanctum* leaves after filtration

After mixing of 1ml of extract with 1mM of $AgNO_3$ and $CuSO_4$ respectively the colour of the solution turns brown and yellow respectively after incubation at room temperature for 2-3 days. The solutions were spectroscopically characterized.

Fig. 8 - 1ml of *O. sanctum* leaves extract + 100ml OF 1mM $AgNO_3$ and $CuSO_4$ for synthesis of NPs

Table 3: Determination of λ max for Cu and Ag NPs

NPs	Wavelength (nm) (λ max)	O.D
Ag NPs	505	0.171
Cu NPs	296	3.173

3) Characterization of NPs: FTIR spectra

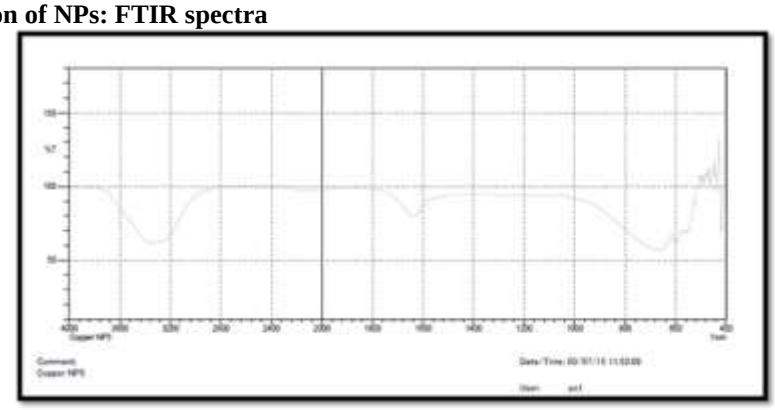


Fig. 9 (a) FTIR spectra of Cu NPs

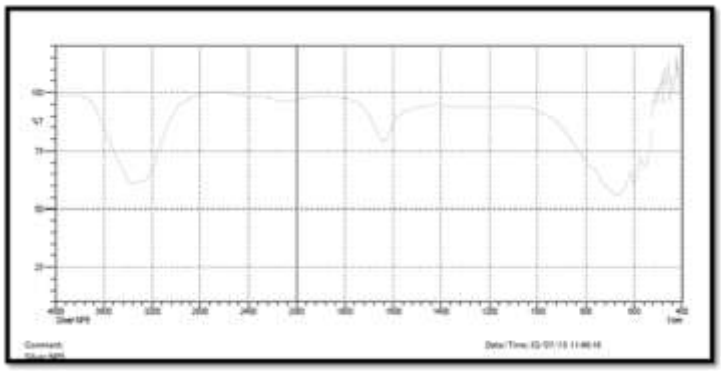


Fig. 10 (b) FTIR spectra of Ag NPs

4) Antimicrobial activity

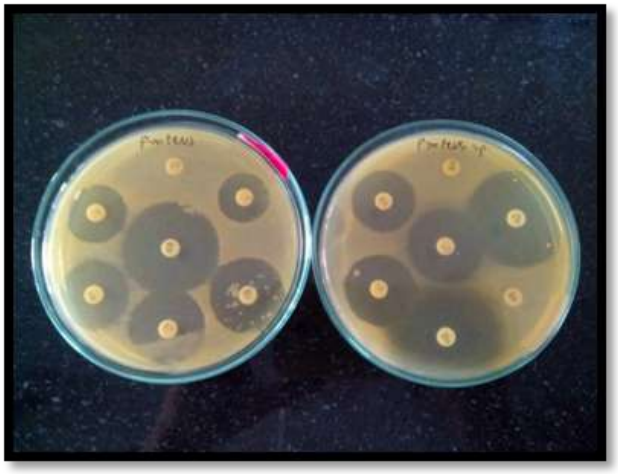
Antimicrobial activity was checked against laboratory cultures like Escherichia coli and Staphylococcus aureus. Both these cultures were inhibited by Cu NPs synthesized microbiologically and Ag and Cu NPs synthesized phytochemically.

5) Confirming the clinical isolates by plating on Selective medium

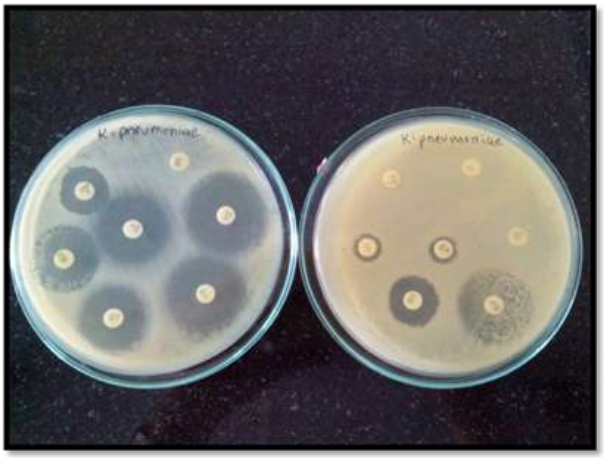
Table 3: growth characteristics on selective plates

Medium	Growth characteristic	Result
BP agar	Brownish black colonies observed	*S. aureus* confirmed
MacConkey' s agar	Pink pinpoint colonies	*E.coli* confirmed
MacConkey' s agar	Pink mucoid big colonies	*K. pneumoniae* confirmed
Cetrimide agar	Bluish colonies	*P. aeruginosa* confirmed
TCBS agar	Yellow colonies	*V. cholerae* confirmed
CLED agar	Bluish green grwoth	*Proteus sp* confirmed
MacConkey' s agar	yellow colonies	*E. faecalis* confirmed

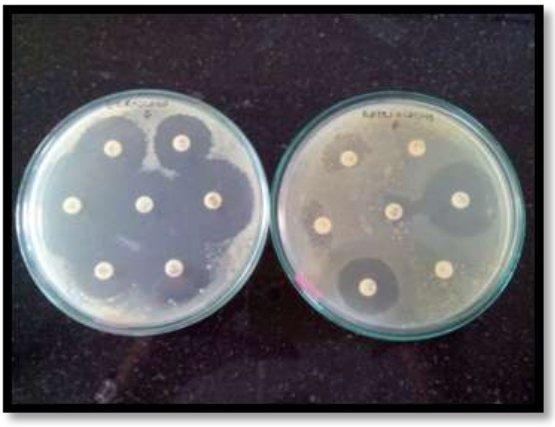
6) Antibiogram of clinical isolates:



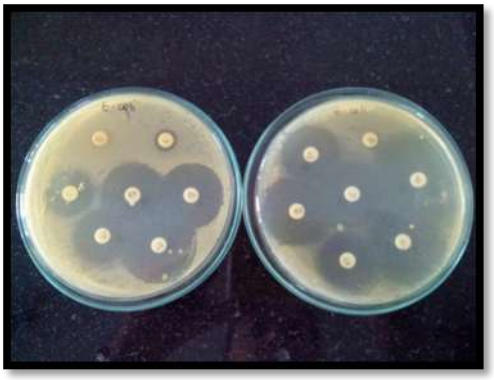
Antibiogram of *Proteus species*



Antibiogram of *K. pneumonia*

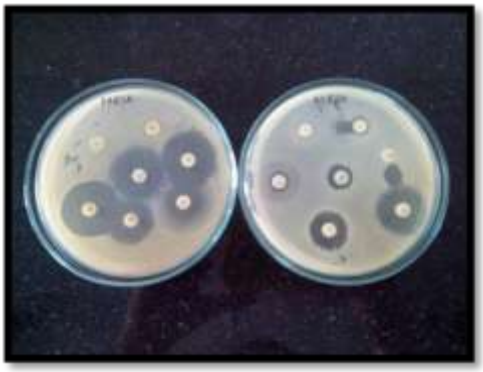


Antibiogram of *E. faecalis*

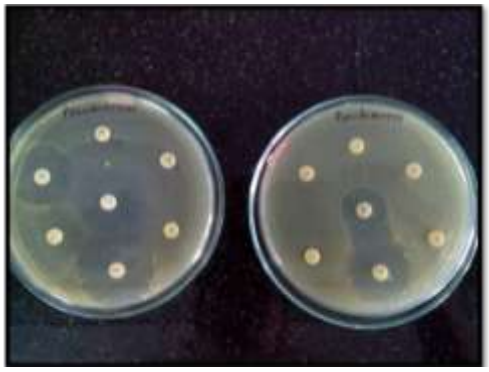


Antibiogram of *E. Coli*

Antibiogram of *V. Cholerae*



Antibiogram of MRSA



Antibiogram of *P. aeruginosa*

Table 4: antibiogram and results of clinical isolates

Antibiotics	Conc (µg)	Zone size (mm)															
		E.c		*P.a*		MRSA		*S.a*		*Prot*		*V.c*		*K.p*		*E.f*	
Gentamycin(G)	30	21	S	26	S	23	S	32	S	24	S	32	S	17	S	30	S
Chloramphenicol(C)	30	23	S	21	S	14	I	22	S	25	S	25.5	S	24	S	22	S
Tetracyclin(TE)	30	21	S	10	R	21	S	31	S	0	R	35	S	19	S	31	S
Amoxycillin(AM)	10	29	S	0	R	7	R	0	R	27	S	27	S	0	R	-	-
Methicillin (MET)	30	0	R	0	R	0	R	19	S	0	R	18	S	0	R	19	S
Vancomycin(V)	30	0	R	0	R	14	S	22	S	0	R	24	S	0	R	22	S
Kanamycin(K)	5	22	S	10	R	12	R	0	R	22	S	0	R	9	R	0	R
Streptomycin(STR)	300	21	S	30	S	22	S	29	S	27	S	26	S	23	S	23	S
Piperacillin(PI)	2	21	S	21	S	12	R	0	R	34	S	27	S	8	R	-	-
Ampicillin(A)	100	10	R	0	R	0	R	0	R	20	S	21	S	0	R	-	-
Cefixime(CFM)	5	26	S	8	R	0	R	29	S	36	S	27	S	11	R	26	S
Amikacin(AK)	10	24	S	28	S	20	S	23	S	23	S	23	S	20	S	25	S
Trimethoprim(TR)	5	32	S	0	R	0	R	0	R	26	S	0	R	23	S	-	-
Levoflaxin (LE)	5	46	S	35	S	24	S	40	S	31	S	38	S	26	S	41	S

7) Antimicrobial activity of NPs

Table 5- Antimicrobial activity of Ag and Cu NPs made using *O.sanctum*

Organisms	Zone of inhibition in mm	
	Ag NPs	Cu NPs
S. aureus	insignificant	insignificant
E. coli	11	-
MRSA	12	12
P. aeruginosa	20	12
Proteus spp	-	-
K. pneumoniae	-	-
V. cholerae	13	-
E. faecalis	-	-

Table 6- antimicrobial activity of Cu NPs made using *P. aeruginosa*

Organisms	Zone of inhibition in mm
S. aureus	22
E. coli	12
MRSA	20
P. aeruginosa	-
Proteus spp	-
K. pneumoniae	15
V. cholerae	20
E. faecalis	15

8 MIC of NPs against clinical isolates:

Table 7- MIC of NPs

Organism	Ag NPs (phytochemically prepared) 1mM		Cu NPs (phytochemically prepared) 1mM		Cu NPs (Microbially prepared) 7mM	
	1 (1mM)	1:2 (0.5mM)	1 1 (1mM)	1:2 (0.5mM)	1 (7mM)	1:2 (3.5mM)
S. aureus	-	+	-	+	-	+
E. coli	+	+	+	+	-	+
Proteus sp	+	+	+	+	+	+
V. cholerae	-	-	+	+	+	+
P. aeruginosa	-	-	+	+	+	+
K. pneumoniae	-	+	+	+	+	+
E. faecalis	-	+	+	+	+	+

IV. Discussions

The aim of this study was to check whether NPs can be synthesized in normal laboratory conditions and whether or not do they possess antimicrobial activity. The NPs were made using a microbial source (*P. aeruginosa*) and a phytochemical source (*O. sanctum).* The cell free extract of *P.aeruginosa* was used for synthesis of Cu NPs and filtrate of *O. sanctum* was used for synthesis of Cu and Ag NPs. The metal solutions used were $AgNO_3$ and $CuSO_4$. The colour changes from blue to green and from colurless to brown respectively and this indicates that metal NPs have formed in the colloidal solution.

Further characterization of these NPs was carried out using UV-Vis Spectroscopy and FTIR. In UV-Vis spectroscopy the range which was obtained for Cu NPs made from *P. aeruginosa* was 300-320nm and the λ max was found to be 310nm due to the excitation of surface plasmon vibrations. The range for Ag NPs made using phytochemical extract was found to be 480 to 520nm and the wavelength at which maximum absorbance

was obtained was 505 nm. The range for Cu NPs was found to be 280-300 nm, which were made using a phytochemical souce. The maximum absorbance found here was 296 nm. Using these data of maximum absorbance, optimization of NPs was carried out. For Cu NPs made by microbial souce the optimum temperature was found to be 27^0C and pH was found to be 7. For Ag and Cu NPs made phytochemically the optimum temperature was 27^0C.

For further characterization, FTIR was also carried out to check the organic groups present around NPs. By studying the FTIR spectra of Cu NPs it is observed that N-H group is present as there is a peak between 3200- 3400cm^{-1}. Also O-H group is present as there is a broad free stretch between 3200-3600cm^{-1}. There is also a C=C group present. For FTIR spectra of Ag NPs it can be observed that there is a peak between 3200-3400cm^{-1} and hence N-H bond is present. With this there is a broad, strong band between 3200- 3600cm^{-1} indicating that there is an 0-H bond present. Also there are various vibrations at 650cm^{-1}, which indiacte that there may be aromatic groups or amino groups present.

Antimicrobial activity of NPs was checked against various clinical isolates. Zone of inhibition is the criteria to check antimicrobial activity. Cu NPs are known to exhibit wide range of antibacterial activity against different strains of gram positive and gram negative bacteria.Cu NPs acts as potential antimicrobial agent against infectious organisms such as *E. coli, B. subtilis, V. cholerae, P. aeruginosa,* and *S. aureus*. (Shobha G, et al; 2014). It was found that Cu NPs were also effective against *Proteus species* and MRSA. The concentration of Cu NPs giving best inhibitions was 10mM.

Cu NPs showed excellent antimicrobial activity against various bacterial strains (*E. coli, P. aeruginosa, K. pneumonia, E. faecalis, P. vulgaris,* and *S. aureus*).Moreover, *E. coli* and *E. faecalis* exhibited the highest sensitivity to CuO NPs while *K. pneumonia* was the least sensitive (Ahamed M, et al; 2013). It was found that *V,cholerae, S. aureus, K. pneumoniae, E. faecalis* were more sensitive as compared *E. coli, Proteus sp, P.aeruginosa* for Cu NPs.

MIC was carried out for NPs. TheMIC is the lowest concentration of the agent that comple tely inhibits visible growth. MIC-determination performed as agar dilution is regarded as the golden standard for susceptibility testing. MIC of Ag NPs for *E. coli, Proteus, V. cholerae* was found to be 0.25 mM, for *P. aeruginosa* and *E. faecalis* MIC was 1 mM concentration. MIC of Cu NPs (synthesized using *P. aeruginosa*) for S. aureus was found to be 5 mM and for E. coli, E. faecalis and V. cholerae was found to be 2.5 mM. MIC of Cu NPs syntheiszed phytochemically for S. aureus was found to be 1mM. For using NPs against these organisms the concentration higher than the MIC can be used.

V. Conclusion

Silver and Copper has always been an excellent antimicrobial and has been used for the purpose for ages and hence prove to be excellent source for synthesis of NPs The rapid biological synthesis of Cu and Ag NPs using cell free extract of *Pseudomonas aeruginosa* and leaf broth of *Ocimum sanctum* provides an environment friendly, simple and efficient route. Chemical and physical methods of Ag and Cu NPs synthesis were being followed over the decades, but they are found to be expensive and the use of various toxic chemicals for their synthesis makes the biological synthesis the more preferred option. Biological synthesis process provides a wide range of environmentally acceptable methodology, low cost production and minimum time required. Here two types of synthesis process were studied (microbial method and phytochemical method).

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