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Bio fabrication of Copper Nanoparticles: A Next Generation Antibacterial Agent against Wound Associated Pathogens

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Abstract

Background/Aim The impaired wound healing is a major complication. Few factors like blood glucose level, poor circulation, Immune System Deficiency, and Infection are the root cause of impaired wound healing. Thus the present study aims at bio-synthesizing copper nanoparticles with potential antibacterial activity against wound associated pathogens.

Materials and Methods: The copper nanoparticles were fabricated utilizing sol gel method with the mixing of *Syzigium cumini* leaf extract in metal salt solution. The particles were then later chararacterized by utilizing UV spectroscopy, SEM, TEM, FTIR, XRD etc. and evaluation for their antibacterial activity and its MIC against four wound associated pathogens.

Results: The results obtained from TEM, SEM and XRD characterization showed the particle size i.e. below 100nm and of spherical shape. The FTIR analysis showed the possibility of various bio molecules which have role in capping and stabilization of the copper nanoparticles. The particles synthesized showed antibacterial activity against four

wound associated pathogens i.e. *P. mirabilis, S. saprophyticus, S. pyogenes,* and *P. aeroginosa.*

Conclusion: The Biosynthesized copper nanoparticles showed potent antimicrobial activity, thus the antibacterial activity of the synthesized copper nanoparticles could be utilized in several biomedical applications additionally they can be exploited as a better therapeutic agent for treating infection seen in impaired diabetic wounds etc. Withal, the particles synthesized by biological route are eco-amicable, less toxic, feasible and cost efficacious.

Key Words

Nanoparticles, Sol–gel process, Biosynthesis, Characterization, Wound associated pathogens, Biomedical applications.

Lists of Abbreviation

UV-VIS Spectroscopy- ultraviolet-visible spectroscopy, FTIR- Fourier transforms infrared spectroscopy, SEM- scanning electron microscope, TEM- transmission electron microscope, *P. mirabilis - Proteus mirabilis, S. saprophyticus- Staphylococcus saprophyticus, S. pyogenes- Streptococcus pyogenes,* and *P. aeroginosa- Pseudomonas aeroginosa*.

1. Introduction

Impaired or delayed wound healing is a major complication seen in various cases specially in diabetic patients.[1,2] There are several factors which are responsible for impaired wound healing, like poor circulation,[3] Diabetic Neuropathy[4,5], Immune System Deficiency,[6,7] Infection and stiffness of the arteries,[8] which lowers the supply of blood, nutrients and oxygen to tissues and ultimately lowers the efficiency of white blood cells to fight against infection.[9] These factors may lead to impaired wound healing, [10,11] thus close monitoring is

very essential.[12] The poor replication of the immune cells is a sign of developing an infection which later more gradual down the rejuvenating process.[13]

From ancient era, metals are known to have good antimicrobial activity, thus in routine lives metal have been utilized for disinfecting water,[14] preservation of victuals,[15] additionally metal coins were utilized by Japanese for dropping into water; milk etc. to treat dysentery during Second World War.[16]

In India Nanobiotechnology is providing an incipient insight in employing Indian greeneries which is a great source of various plant products utilized in Ayurveda for synthesis of eco-cordial and non hazardous nanoparticles.[17] Particle below 100nm is considered as nanoparticles which have unique particle size along with advanced physical, chemical and biochemical properties.[18] Both physical and chemical methods are commercial way of synthesizing metal nanoparticles which are hazardous to the environment, thus there is an essentiality to develop an economically and commercially feasible as well environmentally sustainable route for synthesizing metal nanoparticles to meet demand.[19] These phyto fabrications of metal nanoparticles undergo highly controlled single step protocol with green principles.[20] Phyto constituents present in plant extracts can be utilized to synthesize metal nanoparticles in a single-step.[21] Studies have shown that few metal nanoparticles are kenned to have consequential wound rejuvenating activity.[22] Thus this study may provide insight to methodize nanoparticles synthesis and to provide a direction for future research in impaired wound treatment.

2. Materials and Methods

2.1 Material requirement

Copper sulfate metal salt was purchased from Fisher- Scientific. Nutrient agar and nutrient broth was purchased from Hi media pvt ltd. *P. Aeruginosa* (MTCC No. 3542), *S. Saprophyticus* (MTCC N0. 6155), *S. Pyogenes* (MTCC No. 5969) and *P. Mirabilis* (MTCC No. 3310) are the standard cultures which were procured from Institute of microbial technology, Chandigarh, India. *Syzigium Cumini* leaf extract, Double Distilled water, Ethanol, Magnetic bead, Conical flask, Test tubes etc.

2.2 Method

Preparation of Leaf Extract and Phyto-Chemical Profiling

Syzigium cumini plant leaves were used for the study, which was collected, air dried and then coarsely powdered. Extraction was done utilizing ethanol as a solvent in a soxhlet extractor. The extract was than concentrated. The phytochemical profiling of the plant leaf extract was done utilizing Alkaloids test(Mayer's test), Flavonoids test, Glycosides test, Steroids test(Salkowski's test), Cardiac glycosides test (Keller killiani's test), Saponins test, Resins test, Phenols test (Ferric Chloride Test), Tannins test (FeCl3/ Lead acetate test), Terpenoid test.[23]

Biosynthesis of copper nanoparticles

0.01 M of Copper Sulfate was dissolved in 100 ml of distilled water and then mixed properly by placing it on magnetic stirrer. *Syzigium cumini* leaf extract was utilized for reduction purpose, where plant phytochemicals may itself act as capping agents. Solution was then sanctioned for mixing on magnetic stirrer at a temperature of 500C. After 2 hours sample was accumulated and sanctioned to centrifuge at 14000 rpm. Pellets were collected and then washed thrice by means of ethanol and then kept for drying on dry bath. Sample is then collected and sanctioned for further characterization.

Characterization of copper nanoparticles

The synthesized nanoparticles were characterized by UV-VIS Spectrophotometer, F.T.I.R. Spectrophotometer Model RZX(Perkin Elmer), Scanning Transmission Electron Microscope (SEM) Model JSM6100 (Joel) with Image Analyser, X-ray Diffractometer (Powder Method), and Transmission Electron Microscope (TEM) Hitachi (H-7500).

Antimicrobial activity of copper nanoparticles

Antimicrobial susceptibility testing of bio-synthesized copper nanoparticles was done using Kirby Bauer well diffusion method[24-25] where Mueller Hinton Agar was taken as a medium and the well diameter was kept 5mm and amount of material used was 30µL. 0.5 McFarland standard[26] was used. *P. mirabilis, S. saprophyticus, S. pyogenes,* and *P. aeroginosa* were four different wound associated pathogens against which antimicrobial potential of copper nanoparticles was tested. Solvent blank was utilized as negative control. Pre-existing drug (povidone iodine), Metal salt solution and *Syzigium cumini* plant leaf extract was utilized as a positive control.

Minimum inhibitory concentration of copper nanoparticles

Minimum inhibitory concentration of bio-synthesized copper nanoparticles was then calculated at different concentrations ranging 0.1mg/ml, 0.3mg/ml, 0.5mg/ml, 0.7mg/ml and 0.9mg/ml against *P. mirabilis, S. saprophyticus, S. pyogenes,* and *P. aeroginosa* by broth dilution method in nutrient broth. The concentration of culture was adjusted to 0.2 at 568 nm (1×10^8 CFU/ ml, 0.5 McFarland's standard). Positive and negative control was used as standard. Minimum inhibitory concentration was denoted by analysing the turbidity of the tubes. Small aliquot of the sample (approx 50 µl) from the culture tubes showing least or no

turbidity was taken and poured in agar plate for 24 h at optimum temperature for bacterial growth and was observed for growth if any. Experiment was performed in three sets. [27,28]

3. Results & Discussion

Availability of phyto chemicals in *Syzigium cumini* leaf extract

The qualitative estimation of plant extract was done and the results thus obtained showed the availability of various phyto chemicals which are mentioned in (Table 1).

Nanoparticles synthesis and Visible Observation Changes

There are three main phases of metal nanoparticles synthesis using plant extract i.e. the activation phase which include metal ions reduction and then their nucleation, the second one is the growth phase which include coalesce of small nanoparticles and last one is the termination phase which provide final shape to nanoparticles. Various studies have shown that in the bio-reduction, when metal salt (Copper sulfate) dissolved in distilled water it soon get dissociated into its ionic form i.e. Cu^{2+} and SO_4^{2-}. After mixing of plant extract into the metal salt solution, there is a possibility that the chemical functional groups present within the plant extract interact with metal ions (Cu^{2+}) and reduces it to its zerovalent state (Cu^0) thus leads to the formation of metallic copper nuclei followed by growth phase, leaving rest of the components as by-product.[29]

Thus addition of plant extract convert the bulk copper to copper nanoparticles leaving the by product aside and ultimately changes in the colour of the solution. After integration of plant extract to metal salt solution the colour of the copper sulfate salt solution turn from bluish-greenish to brownish- reddish in colour which can be seen in (Figure 1A & 1B). Bio-reduction and bio-sorption are two major steps required for nanoparticles synthesis, by using various phyto products like plant phytochemicals, carboxylic and amino groups, proteins and

carbohydrates.[30] The colorimetric changes given by nanoparticles are due to the property of quantum confinement which is a size dependent property of nanoparticles which affects the optical property of the nanoparticles.[31-33] Thus the resulting colour change is may be due to the quantum confinement property of copper nanoparticles.

UV Spectroscopy

Applied electromagnetic field causes the excitation of surface Plasmon present on periphery of nanoparticles which leads to occurrence of the phenomena called surface Plasmon resonance.[34] The range UV absorption apex of copper nanoparticles is 573–600 nm.[35-36] The result obtained from UV-Vis spectra (Figure 2) showed the absorption peak approximately at 582 nm indicating the formation of copper nanoparticles. An additional peak of 558nm was also obtained. A broad absorption peak at 582 nm is due to the surface plasmon resonance absorption band along with free electronic vibrations of copper nanoparticles in resonance with a light wave.[37]

FTIR Spectroscopy

Fourier Transform Infrared spectroscopy is a characterization technique which is utilized to quantify the vibration frequencies mentioned in Table 2. of bonds in the molecule which can be seen in (Figure 3) The FTIR analysis is done to understand the vibrational kinetics of atoms or molecules identify the possible phytoconstituents responsible for the reduction as well as capping of reduced copper nanoparticles along with the nature of surface adsorbents.[38-40] The alternate modification by such adsorbents (Functional groups) may generate different properties. The FTIR Spectra due to such adsorbents over surface of nanoparticles thus showed a number of absorption peaks, each peak is designating the availability of particular functional groups present in the plant extract.[41] It is thus possible to understand the oxidation levels of synthesized nanoparticles prepared at different partial

oxygen pressures. From FTIR data it is possible to study the oxidation levels of nanoparticles prepared at different partial oxygen pressures.[42]

Previous studies have shown that terpenoids are oftenly associated with nanoparticles as analysed in FTIR spectroscopy results. Also terpenoids has an essential role in transforming metal ions into nanoparticles.[43] by dissociating eugenol OH-group proton thus generating structures that can be further oxidized, it leads to reduction of metal ions, and ultimately formation of nanoparticles.[44] Flavonoids Tautomeric shift i.e. from enol to keto results in releasing of reactive hydrogen resulting in reduction of metal ions and leads to nanoparticles formation.[45] In plant sugars, by means of nucleophilic addition of OH-, oxidization of aldehyde group to carboxyl group occurs, which leads to metal ion reduction and nanoparticles synthesis.[46] Similarly different functional groups have different mechanism for nanoparticles synthesis. The knowledge about the exact mechanism behind nanoparticles synthesis is still unknown and this area thus needs further exploration.

XRD Analysis

X-Ray Diffraction patterns of copper nanoparticles were recorded by utilizing X-ray Diffractometer (Powder Method) which can be optically discerned in (Figure 4). Debye-Scherer's equation i.e. $D = 0.9\lambda/ \beta \cos\theta$, was habituated to calculate the size of copper nanoparticles, where D represents crystalline size, λ represents wavelength of X-ray, β represents full width at half maximum of the diffraction peak and θ represents Bragg's angle. At 2θ values, a number of Bragg reflection peaks were observed at 26.79, 32.4, 35.5, 36.4, 44.1, 48.7, 50.6, 58.3 and 75.6 which were indexed to (111), (110), (002), (111), (200), (202), (200), (202) and (220) crystallographic planes of face-centred cubic (FCC), (JCPDS, File No. 04-0836 and JCPDS No.45-0937). Additional peaks obtained seen during XRD analysis like 35.09, 35.90 and 36.52 revealed the availability of CuO nanoparticles and 47.49 revealed the

availability of Cu_2O nanoparticles which may have occurred due to exposure of the nanoparticles with surrounding environment during characterization. The particle size estimated is below 100 nm (Calculated by Debye-Scherer's equation). The width of the peaks obtained in XRD pattern is cognate to the crystallite size of the particle.[47] The small size of the nanoparticles synthesized thus increases their high surface area, and surface area to volume ratio.[48]

TEM & SEM Analysis

The synthesized nanoparticles are of spherical or ellipsoidal symmetry. The sizes of the copper nanoparticles were below 100nm which can be seen in (Figure 5). The SEM analysis reveals the shape of the synthesized nanoparticles which is roughly spherical or ellipsoidal and it withal revealed the size of the synthesized nanoparticles which is below 100nm. The obtained result supports the result obtained from TEM analysis which can be seen in (Figure 6). The result obtained from TEM and SEM study showed the presence of copper nanoparticles i.e. below 100nm.

Antimicrobial activity of copper nanoparticles:

Antimicrobial activity of copper nanoparticles revealed that copper nanoparticles has consequential antibacterial activity against wound associated pathogens as compared to the plant extract and pre-subsisting drug (povidone iodine) which can be seen in (Figure 7A-H). Biosynthesized copper nanoparticles exhibited good antibacterial activity against *P. mirabilis, S. saprophyticus, S. pyogenes,* and *P. aeroginosa* i.e. 16mm, 15mm, 14mm and 12mm respectively. Whereas Biosynthesized copper nanoparticles + pre-subsisting drug (povidone iodine) exhibited good antibacterial activity against *P. mirabilis, S. saprophyticus, S. pyogenes,* and *P. aeroginosa* i.e. 20mm, 17mm, 18mm and 14mm respectively. In integration to that povidone iodine exhibited less activity against *P. mirabilis, S.*

saprophyticus, and *S. pyogenes*, i.e. 11mm, 8mm, 8mm but no activity against *P. aeroginosa.* Moreover Metal salt solution withal exhibited less activity against *P. mirabilis* and *S. saprophyticus*, i.e. 10mm, 9mm and no activity against *S. pyogenes* and *P. aeroginosa*.

Minimum inhibitory concentration and minimum bacterial concentration of copper nanoparticles

The minimum inhibitory concentration and minimum bacterial concentration of copper nanoparticles was evaluated by analysing the turbidity of culture tubes. Culture tubes containing nanoparticles ranging from 0.1mg/ml to 0.5mg/ml showed bacterial growth, whereas no growth was seen in culture tubes containing nanoparticles 0.7mg/ml and 0.9mg/ml. Little aliquot of the sample poured in agar plated showed no bacterial growth when allowed to grow for 24 hours at optimum temperature condition, showing bactericidal property of copper nanoparticles at this particular concentration. Thus it can be concluded that both minimum inhibitory concentration and minimum bacterial concentration of copper nanoparticles is effective at concentration of 0.7mg/ml.

Antibacterial activity results revealed that copper nanoparticles and copper nanoparticles + Pre-existing drug acted as potent antibacterial agents against wound associated pathogens when compared to pre-subsisting drug (povidone iodine), Copper sulfate salt solution and Plant extract utilized for nanoparticles synthesis. The potential antimicrobial activity of the synthesized copper nanoparticles is may be due to grain size of nanoparticles having high surface to volume ratio.

Nanoparticles are known to have killing activity and lowering of microbial, where the surrounding tissue remains unaffected.[49] The mode of action of antimicrobial agent is in two ways either they act as bactericidal, or they may be bacteriostatic.[50] The antibacterial property of antibacterial agents can be used to fight infectious diseases by reducing the bacterial load.

There is a significant difference between strains of bacteria, thus the use of antibacterial agent should also be specific to respective strains. Nanoparticles thus exert toxic effect against bacteria.[51] There are several factors that affect the antimicrobial activity of nanoparticles against various microbial species. Some of them are discussed here:

The cell wall protects the cell from various damages and ruptures as they provide stability, protection, rigidity, and shape to the cell. Tolerance as well as susceptibility is not only dependent on the structure of the cell wall, there are several factors which affect the tolerating ability as well as susceptibility to nanoparticles like bacterial growth rate and biofilm formation.[52]

The bacterial growth rate is another factor that affects the tolerance of bacteria against nanoparticles. Susceptibility of fast-growing bacteria is more for nanoparticles and antibiotics as compared to slow-growing bacteria (in relation to the expression of stress-response genes).[53]

Formation of biofilm (adhesion of microbial species to a solid surface together with matrix secretion covering them) by bacteria is a major drawback for antibacterial drugs as well as for nanoparticles to fight against bacteria. The interaction of biofilm as well as nanoparticles is dependent on their electrostatic properties.[54]

5. Conclusion

The plant leaf extract we have used showed great capability to synthesis copper nanoparticles at optimum temperature conditions. The UV absorption peak at 582.00 nm designates the synthesis of copper nanoparticles. The SEM and TEM studies were used with the aim at deciphering the morphology and size of the particle. FTIR studies showed the bio fabrication of the copper nanoparticles by the action of different phyto-chemicals with its different

functional groups present in the extract solution. The XRD patterns showed the purity, phase composition and nature of the synthesised nanoparticles. The following study justified the synthesis of stable nanoparticles, which could be due to the presence of capping and stabilizing materials such as flavonoids and terpenoids within the plant extract.

Additionally the bio-synthesized copper nanoparticles have shown potential antimicrobial activity against four different wound associated pathogens as compared to the pre-subsisting drug povidone iodine. Thus the present work focuses on highlighting approaches of bio reduction approaches to synthesize copper nanoparticles utilizing Plant extract and antibacterial activity of synthesized nanoparticles. Various studies have already reported for the synthesis of metal nanoparticles utilizing physical and chemical methods but the methods generally employ utilization of rigorous chemicals and stringent protocol which is hazardous to the environment. Thus it is a prerequisite to develop a protocol which is simple, cost-efficacious, eco cordial, facile scale up. The exact mechanism of metal nanoparticles synthesis utilizing plant products is still not clear but there are several studies which somehow focus on the possible mechanisms behind it. Bio-reduction and bio-sorption are two major steps required for nanoparticles synthesis, governed by utilization of various phyto products like plant phyto-chemicals, carboxylic and amino groups, proteins and carbohydrates. Thus the study may support the proper wound management with special reference to antimicrobial activity of bio fabricated copper nanoparticles.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1: The qualitative estimation of Phyto constituents in *Syzigium cumini* leaf extract

S.No.	Phyto constituents	Availability in Ethanol extract
1.	Flavonoids	+
2.	Alkaloids	+

3.	Glycosides	+
4.	Steroids	+
5.	Phenols	+
6.	Terpenoid	+
7.	Saponins	-
8.	Resins	+
9.	Tannins	+
10.	Cardsiac glycosides	-
11.	Phytosterols and Triterpenoids	+
12.	Carbohydrates	+
13.	Fixed oils and fats	-

Table 2: Vibrational frequencies of functional groups of possible phytoconstituents obtained by FTIR analysis

S. no.	**Frequency (cm^{-1})**	**Possible functional groups**
1.	3377.6 cm^{-1}	O-H stretch vibration of Phenols
2.	1632.12 cm^{-1}	N-H bend of primary amines
3.	1514.13 cm^{-1}	N-O asymmetric stretch vibration of nitro compounds

4.	1198.6 cm^{-1}, 1117.3 cm^{-1} and 1107.3 cm^{-1}	C-N stretch vibration of aliphatic amines
5.	864.11 cm^{-1}	N-H bond of primary and secondary amines
6.	803.11 cm^{-1},676.10 cm^{-1} and 626.8 cm^{-1}	C-Cl stretch vibration of alkyl halides
7.	594.9 cm-1	Cu-O Stretching vibration

Table 3: Bacterial growth at different concentration of copper nanoparticles

Dilution for same bacterial concentration	**Sets**	**Bacterial growth at different concentrations of copper nanoparticles**				
		0.9mg/ml	**0.7mg/ml**	**0.5mg/ml**	**0.3mg/ml**	**0.1mg/ml**
P. mirabilis	Set 1	-	-	-	+	+
	Set 2	-	-	-	+	+
	Set 3	-	-	-	+	+
S. saprophyticus	Set 1	-	-	+	+	+

	Set 2	-	-	-	+	+
	Set 3	-	-	-	+	+
S. pyogenes	Set 1	-	-	-	+	+
	Set 2	-	-	-	+	+
	Set 3	-	-	+	+	+
P. aeroginosa	Set 1	-	-	-	+	+
	Set 2	-	-	+	+	+
	Set 3	-	-	+	+	+

+ = Turbidity due to microbial growth; - = No turbidity

Table 4: Minimum Bactericidal Concentrations of copper nanoparticles

Dilution of copper nanoparticles	**Sets**	**Different concentration of copper nanoparticles**	
		0.9mg/ml	**0.7mg/ml**
P. mirabilis	Set 1	-	-
	Set 2	-	-
	Set 3	-	-
S. saprophyticus	Set 1	-	-

	Set 2	-	-
	Set 3	-	-
S. pyogenes	Set 1	-	-
	Set 2	-	-
	Set 3	-	-
P. aeroginosa	Set 1	-	-
	Set 2	-	-
	Set 3	-	-

+ = Bacterial growth; - = No bacterial growth

Fig. 1A: Visible observation of Copper sulfate salt solution before adding plant leaf extract

Fig. 1B: Visible observation of Copper sulfate salt solution after adding plant leaf extract

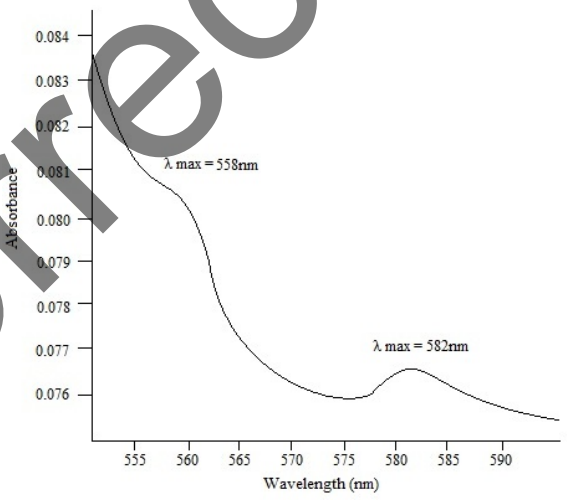

Fig. 2: UV analysis of copper nanoparticles

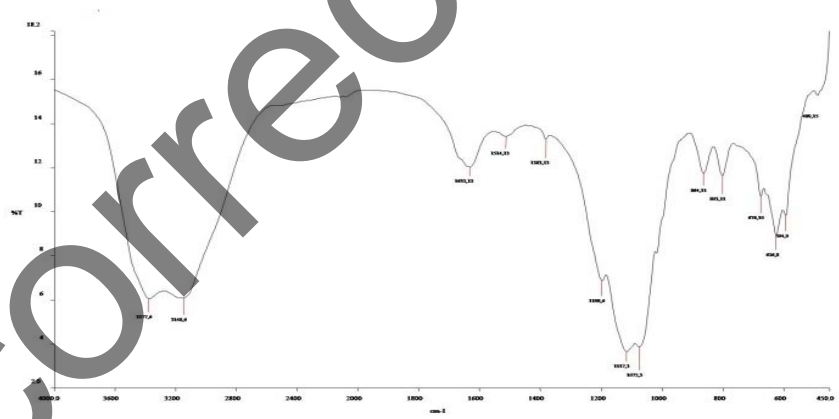

Fig. 3: FTIR analysis of copper nanoparticles

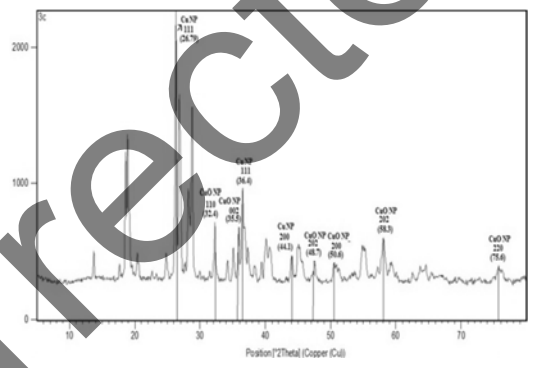

Fig. 4: XRD analysis of copper nanoparticles

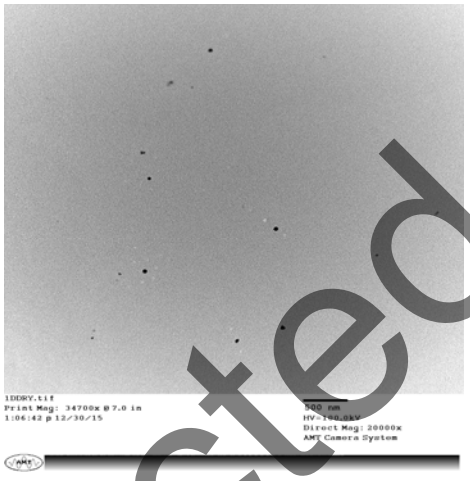

Fig. 5: TEM analysis of copper nanoparticles (below 100 nm)

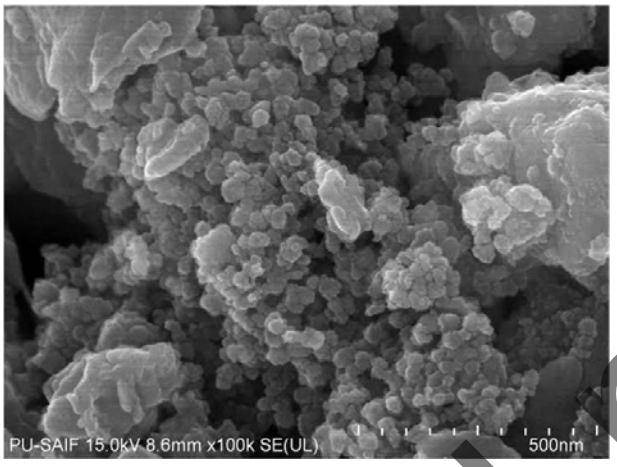

Fig. 6: SEM analysis of copper nanoparticles

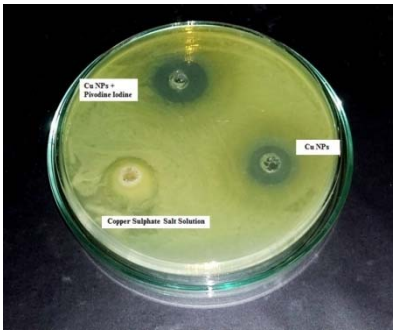

Fig. 7A: Antibacterial activity of copper nanoparticles against *P. mirabilis*

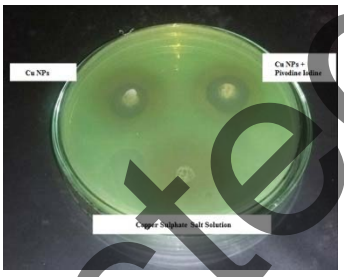

Fig. 7B: Antibacterial activity of copper nanoparticles against *S.saprophyticus*

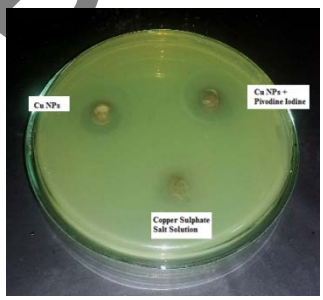

Fig. 7C: Antibacterial activity of copper nanoparticles against *S. pyogenes*

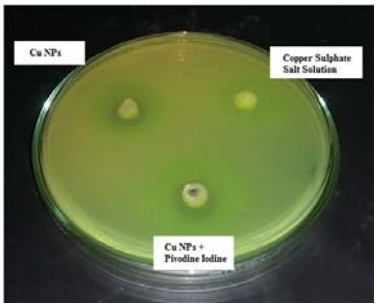

Fig. 7D: Antibacterial activity of copper nanoparticles against *P. aeroginosa*

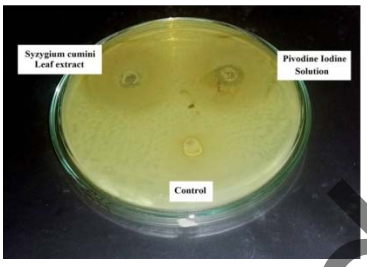

Fig. 7E: Antibacterial activity of Plant leaf extract and povidone iodine against *P. mirabilis*

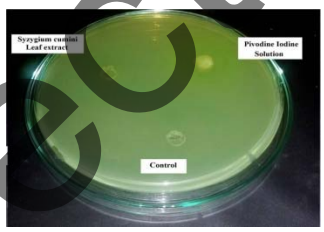

Fig. 7F: Antibacterial activity of Plant leaf extract and povidone iodine against *S. saprophyticus*

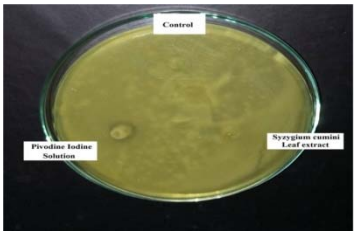

Fig. 7G: Antibacterial activity of Plant leaf extract and povidone iodine against *S.pyogenes*

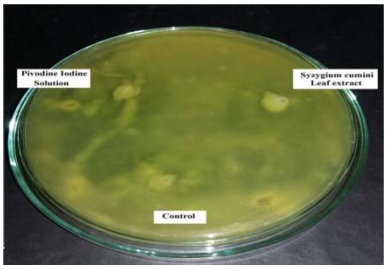

Fig. 7H: Antibacterial activity of Plant leaf extract and povidone iodine against *P. aeroginosa*