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BIOSYNTHESIS OF COPPER NANOPARTICLES USING *ANNONA MURICATA* LEAF EXTRACT-CHARACTERIZATION AND THEIR ANTIBACTERIAL ACTIVITY

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ABSTRACT

Vegetable mediated synthesis of nanoparticles is a green chemistry approach that connects nanotechnology and biotechnology. In the present investigation, we have used a fast, convenient and environment friendly method for the synthesis of copper nanoparticles by biologically reducing copper nanoparticles with aqueous extract of *Annona Muricata* under optimum conditions (pH 7.2). The formation of copper nanoparticles was indicated by the colour change from blue to pale brown. Biosynthesized nanoparticles were characterized by UV-Vis, XRD, SEM, Particle size analysis, Zeta potential analysis and FT-IR analysis. These biologically synthesized copper nanoparticles were tested for antimicrobial activity against three human pathogens viz, *E.Coli*, *Klbesiella pneumoniae* and *Staphylococcus aureus.*

KEYWORDS: *Annona Muricata*, UV-Vis, XRD, SEM, FT-IR and Antimicrobial activity.

INTRODUCTION

The word “nano” is used to indicate one billionth of a meter or 10^{-9}. Nanoparticles are clusters of atoms and their size from 1–100 nm. “Nano” is a Greek word meaning extremely small.[1] Nanotechnology is a field of science which deals with production, manipulation and use of materials ranging in nanometers.[2] Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has driven research toward a multitude of potential uses for Nanomaterials.[3]

Nanotechnology is considered an emerging technology due to the possibility to advance well established products and to create new products with totally new characteristics and functions with enormous potential in a wide range of applications. In addition to various industrial uses, great innovations are foreseen in Information and Communication Technology, Biology and Biotechnology, Medicine and Medical Technology, in Metrology, etc. It is anticipated that Nanotechnology can have an enormous positive impact on human health.[4]

Copper nanoparticles, due to their excellent physical and chemical properties and low cost of preparation, have been of great interest. Copper nanoparticles have wide applications as heat transfer systems, antimicrobial materials, super strong materials, sensors and catalysts. Copper nanoparticles are very reactive because of their surface-to-volume ratio and can easily interact with other particles and increase their antimicrobial efficiency.[5]

A. muricata L., commonly known as soursop, graviola, guanabana, paw-paw and sirsak, is a member of the Annonaceae family comprising approximately 130 genera and 2300 species.[6] The basic photochemical screen has revealed *Annona muricata* to contain saponins, glycosides, tannins and flavonoids.[7] The leaves of *Annona muricata* have essential oils with parasiticidal, anti-diarrheal, rheumatological and anti-neuralgic properties.[8] Phytochemical screening of the leaves of *A. Muricata* has shown it to consist of alkaloids such as reticuline, coreximine, coclarine and anomurine[9], annomuricin E, annomuricin C, muricatocin C, gigantetronenin and muricapentocin with antioxidant and antitumor properties[10], as well as, essential oils such as β-caryophyllene, δ-cadinene, epi- α-cadinol and α-cadinol.[11]

The green synthesis method have several advantages over other methods namely cost effectiveness, simplicity, use of less temperature, the usage of less toxic materials, moreover it is compatible for medical and food applications.[12,13] Many researchers used green synthesis methods for different metal nanoparticles due to their growing need of eco-friendly properties.[14] Green synthesis method was found to be the best method when compared to the other method such as chemical reduction, photochemical reduction, electrochemical reduction, heat evaporation etc.[15] In this method, the plant extract has been used as capping and reducing agent for the synthesis of copper nanoparticles due to their reducing properties present in the leaf extract.[16]

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Annona Muricata leaves were collected from the Coimbatore local market. They were washed and cleaned with distilled water. Thoroughly washed leaves (100 g) were cut and boiled with 100 ml of deionised water for 15 min in heating mental at temperature 80°C. The resulting product was filtered and stored in refrigerator for further experiments.

SYNTHESIS OF COPPER NANOPARTICLES

For the Cu nanoparticles synthesis, 1 ml of *Annona Muricata* leaf extract was added to 100 ml of 1mM (0.1596 g in 100ml) aqueous $CuSO_4.5H_2O$ solution in a 250 ml Erlenmeyer flask. The flask was then kept overnight at room temperature. The Cu nanoparticles solution thus obtained was purified by repeated centrifugation at 12,000 rpm for 15 min followed by re-dispersion of the pellet in deionised water. Then the Cu nanoparticles were dried in oven at 80°C. After the drying the nanoparticles were collected in microfuge tube for further characterization techniques.

CHARACTERIZATION TECHNIQUES

The formation of copper nanoparticles was confirmed by UV- Visible spectroscopy using Jasco V-550 spectrophotometer instrument. Size of the Cu-NPs was analysed with UV-Spectrometer in the range between 300-700nm.

The crystalline structure of the copper nanoparticles were determined by X-Ray diffraction analysis using Rigaku X-Ray diffractometer (Miniflex, UK) instrument operating at 40 kV with 2sec time interval at room temperature 27ºC. Morphology and mean particle size of the Cu were determined by SEM analysis. The samples were prepared for SEM analysis.

The particle size distribution of the powder was measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano ZS laser particle size analyzer. The instrument was equipped with a He-Ne laser source (=633 nm) and at scattering angle of 1730. The dispersion concentration was around 0.1 g/L. The suspension was prepared by dispersing the powder in distilled water and treated for 6mins in an ultrasonic bath to obtain a well-dispersed suspension. The zeta potential measurements were also performed using a Zeta sizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable

FTIR analysis was carried out using using Jusco 5300 model FTIR instrument.

ANTIBACTERIAL ACTIVITY

Antibacterial activity of the extract was determined on Nutrient Agar (Hi-Media Pvt. Ltd .Mumbai) using disk well diffusion method. Test pathogens were spread on the test Nutrient Agar using sterile swabs. Sterile wells are made with the help of a sterile cork borer at aseptic conditions. Samples were added to the wells at aseptic conditions. Stock solutions of the extract were prepared using DMSO. The test plates were incubated for 24hrs. The zone of inhibition (in mm diameter) were read and taken as the activity of the extract against the test organisms.

ANTIFUNGAL ACTIVITY

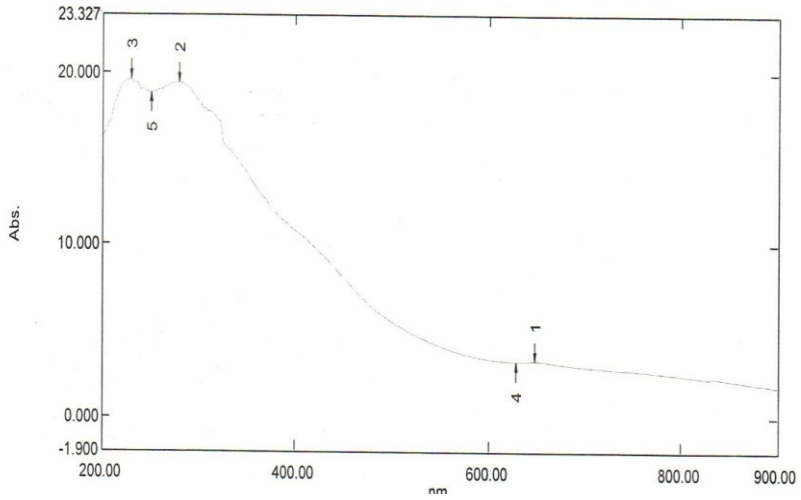
The *in vitro* antifungal activity of plant extract was performed according to the method Abu-Jawdah with a minor modification in dilution and instead of test tubes, petriplates were used.[17,18] The final concentration of the sample in the petri-plates ranged from 100, 50 and 25 µg/ mL from the 1st to 3rd plate respectively. About 1µL of standardized fungal spore suspension ($1x10^7$ spores/mL) was carefully liquidated using micropipettes at the center of each petriplate amended with copper nanoparticle in different concentration and allowed to diffuse in the media. The antifungal agent Fluconazole (1µg/mL) and 2 mL of 2% DMSO was used as positive and negative assay controls respectively. The plates were incubated at 27 ±2°C for 7days and fungal growth (mm) in each plate was measured and averaged. The assay was carried out in triplicates to attain statistical significance and fungal growth inhibition percentage was calculated with reference to the negative control by applying a formula described.

RESULTS AND DISCUSSION

CHARACTERIZATION TECHNIQUES

UV-Vis SPECTROSCOPY

In UV-VIS spectroscopy, the absorption peaks due to the surface plasmon resonance (SPR) were observed at 279 nm and 230nm which attributed the preparation of Cu-NPs. The peaks so obtained at 279 nm & 230 nm respectively account for the polyphenolic groups present in the *Annona Muricata* leaf extract (Fig.1). *Annona Muricata* leaf represents a complex storehouse of myriad of biomolecules like ascorbic acid, vitamin A, flavonoid fractions, including Hesperidins, Neohesperidin, and Diosmin and various other Polymethoxylated Flavones like Nobiletin and Tangeritin (rarely found in other plants) and the peaks obtained in these regions can be accounted due to the presence of the above mentioned compounds.



No.	P/V	Wavelength	Abs.	Description
1	↑	648.00	3.306	
2	↑	279.00	19.424	
3	↑	230.00	19.591	
4	↓	629.00	3.271	
5	↓	251.00	18.761	

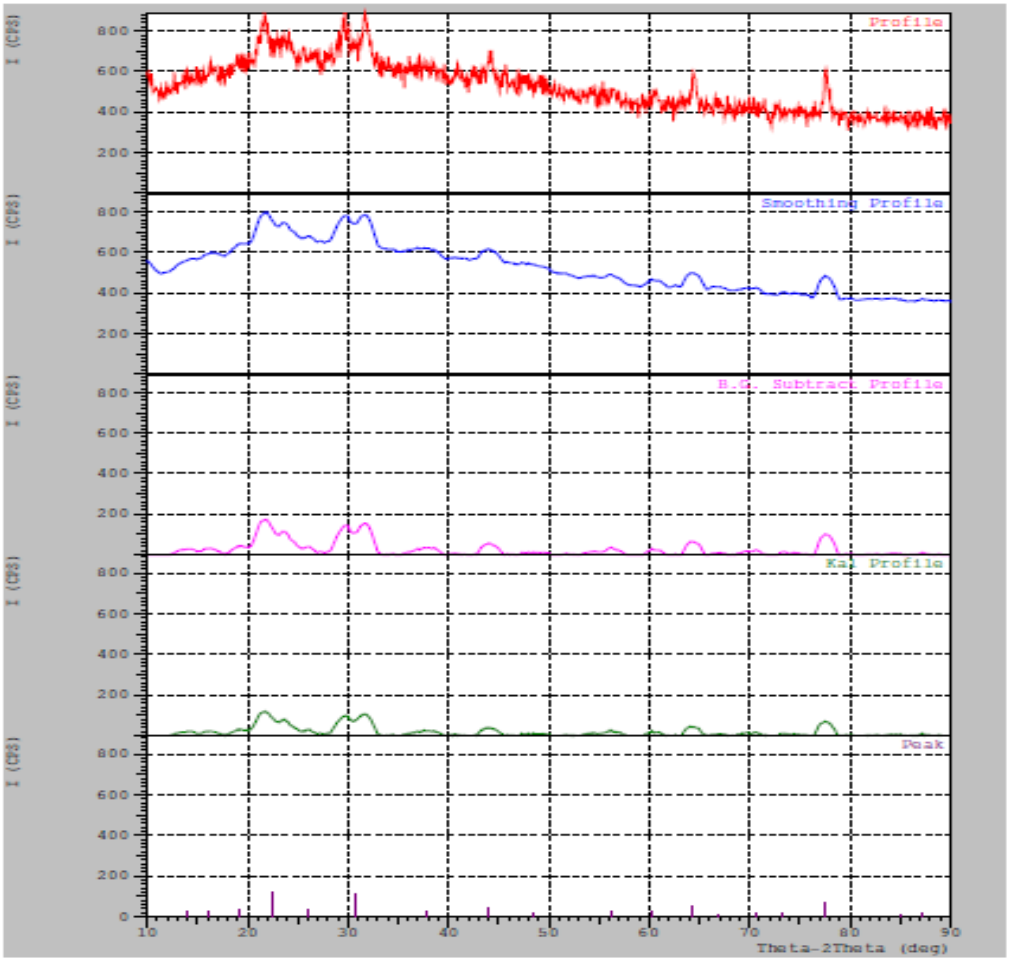
Fig.1: UV-Vis Spectroscopy

X-Ray DIFFRACTION

XRD pattern of synthesized Cu nanoparticles using a leaf extract of *Annona Muricata* was shown in (Fig.2). The XRD pattern Showed crystallinity of Cu nanoparticles with diffraction angles of 22.4°, 30.6° and 77.5°, which correspond to the characteristic face centered cubic of copper lines indexed at (100), (100) and (220), respectively(Table.1). The size of the nanoparticles obtained were estimated to be 0.0409nm, 0.0384nm and 0.1123nm using Debye-Scherrer Equation, which may indicate a high surface area and surface area to volume ratio of the nano-crystals, The equation is written below

$$d = \frac{K\lambda}{\beta \cos(\theta)}$$

Where K, known as Scherer's constant(shape factor), ranges from 0.9 to 0, λ is 1.5418 Å which is the wavelength of the X-Ray radiation source, β1/2 is the width of the XRD peak at half height and θ is the Bragg angle.



Strongest 3 peaks

no.	peak no.	2Theta (deg)	d (A)	I/I1	FWHM (deg)	Intensity (Counts)	Integrated Int (Counts)
1	4	22.4128	3.96359	100	3.45430	72	2143
2	6	30.6458	2.91495	89	3.74170	64	2202
3	16	77.5750	1.22966	60	1.58340	43	688

Fig.2: X-Ray Diffraction

Table.1: The grain size of Cu-Nano powder

S. No	2θ	hkl	θ	FWHM	Size of the particle	d-Spacing
1	22.41	100	11.20	3.4543	2.45nm	0.3967
2	30.64	100	15.32	3.7417	2.3nm	0.2916
3	77.57	220	38.78	1.5834	6.74nm	0.1230

SCANNING ELECTRON MICROSCOPY (SEM)

The surface morphology and size of the nanoparticles were observed by Scanning Electron Microscopy (SEM) analysis. The fig.3 shows the Cu-NPs synthesized by the plant extract of *Annona Muricata.* The electrostatic interactions and hydrogen bond between the bio-organic capping molecules bond are responsible for the synthesis of copper nanoparticles using plant extract.

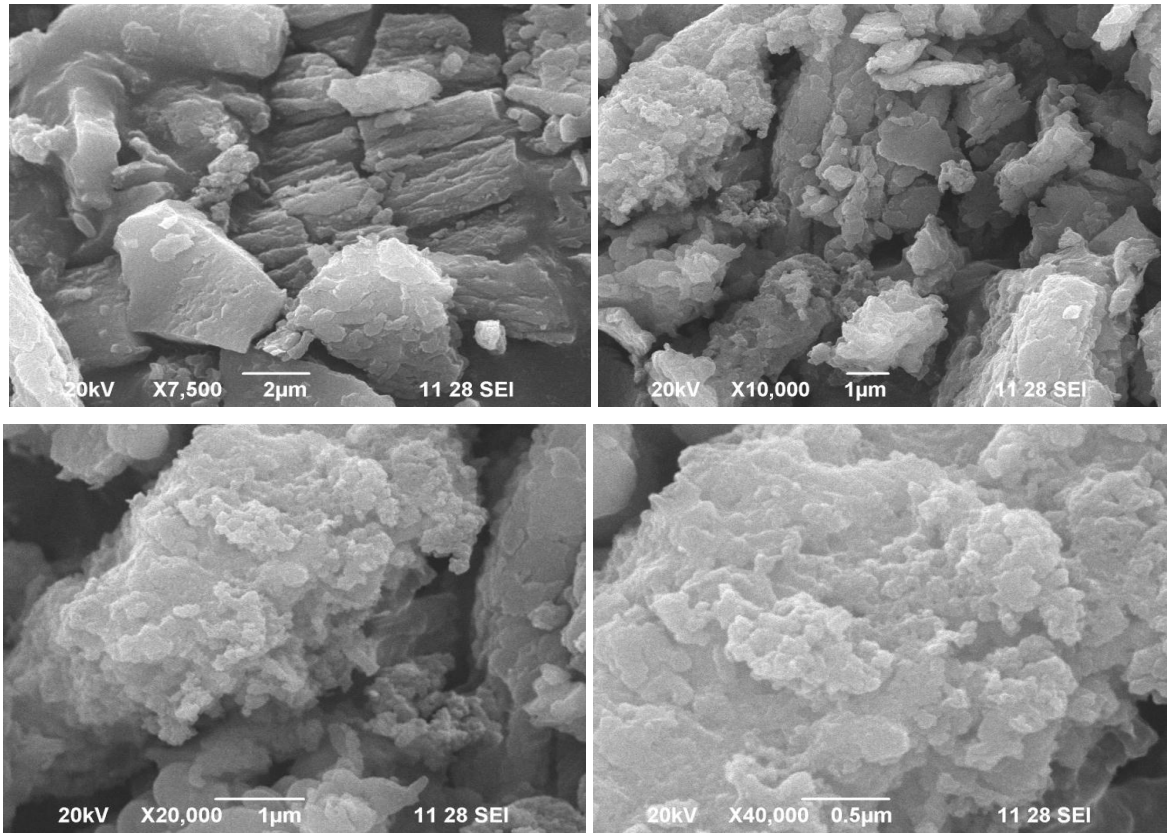


Fig.3: Scanning Electron Microscopy

PARTICLE SIZE ANALYSIS AND DISTRIBUTION

Dynamic light scattering (DLS) results are often expressed in terms of the Z-average. The Z-average arises when DLS data is analyzed by the use of the technique of cumulants Z-average size increases as the particle size increases. Therefore it provides a reliable measure of the average size of a particle size distribution (Fig.4).

Particle size and distribution are the most important characteristics of nanoparticles. They determine the *in vivo* distribution, biological fate, toxicity and the targeting ability of the nanoparticle systems. Though the exact reason is yet unknown, it has been observed that smaller the size of the nanoparticles, greater is their antibacterial activity. Hence the synthesized nanoparticles were tested for their size distribution using the HORIBA Partica analyzer which uses the principle of dynamic light scattering (DLS to test the samples. The size distribution of the Cu-NPs was within 216 nm.

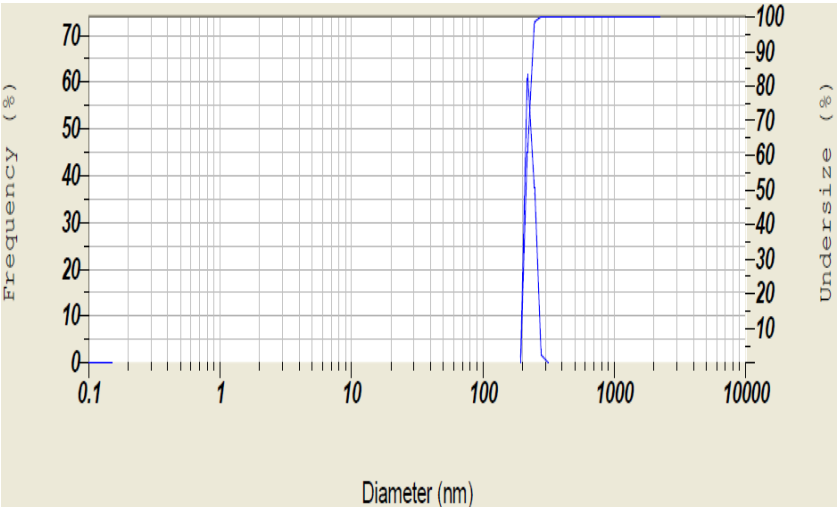


Fig.4: Particle Size Analysis

ZETA POTENTIAL ANALYSIS

The stability of the *Annona Muricata* Cu-NPs was determined by measurement of zeta potential. As shown in the Fig.5 the Cu-NPs obtained possess a negative zeta potential value. A minimum zeta potential value is required for indication of stable nano-suspension. The zeta potential value for *Annona Muricata* Cu-NPs is -13.5 mV. Nanoparticles with Zeta Potential values greater than +25 mV or less than -25 mV typically have high degrees of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Wal inter-particle attractions.

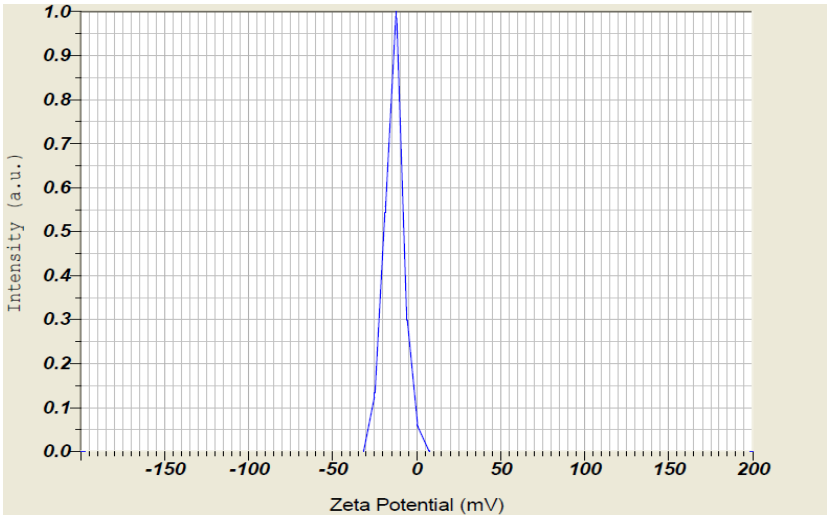


Fig.5: Zeta Potential Analysis

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The FTIR spectrum of Cu nanoparticles was shown in Fig.6. The IR spectrum of Cu nanoparticles shown band at 3256.99 cm-1 , 2919.52cm-1 , 1633.27 cm-1, 1061.29 cm-1 and

678.16 cm-1 corresponds to O-H stretching H-bonded alcohols and phenols, aldehydic C-H stretching Alkanes, -NH2 bendind, corresponds to C-O stretching alcohols and esters and bending vibrations of amines and amides respectively. FTIR spectrum of Cu nanoparticles suggested that Cu nanoparticles were surrounded by different organic molecules such as terpenoids, alcohols, ketones, aldehydes and carboxylic acid.

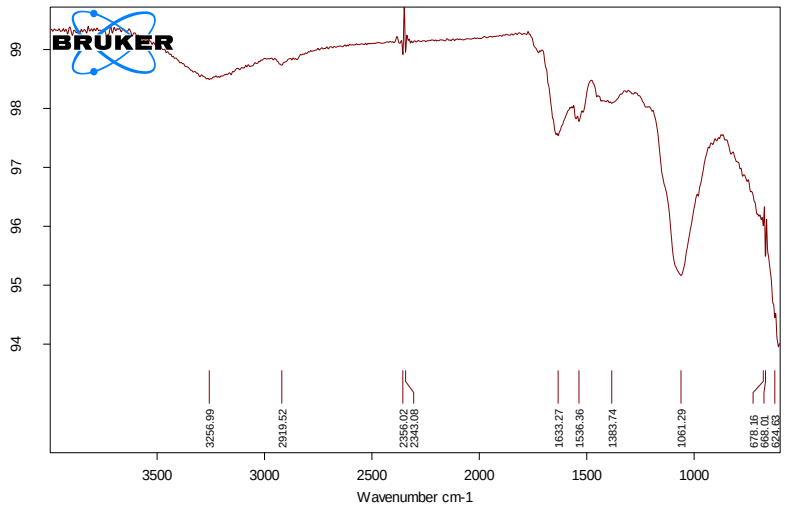


Fig.6: Fourier Transform Infrared Spectroscopy

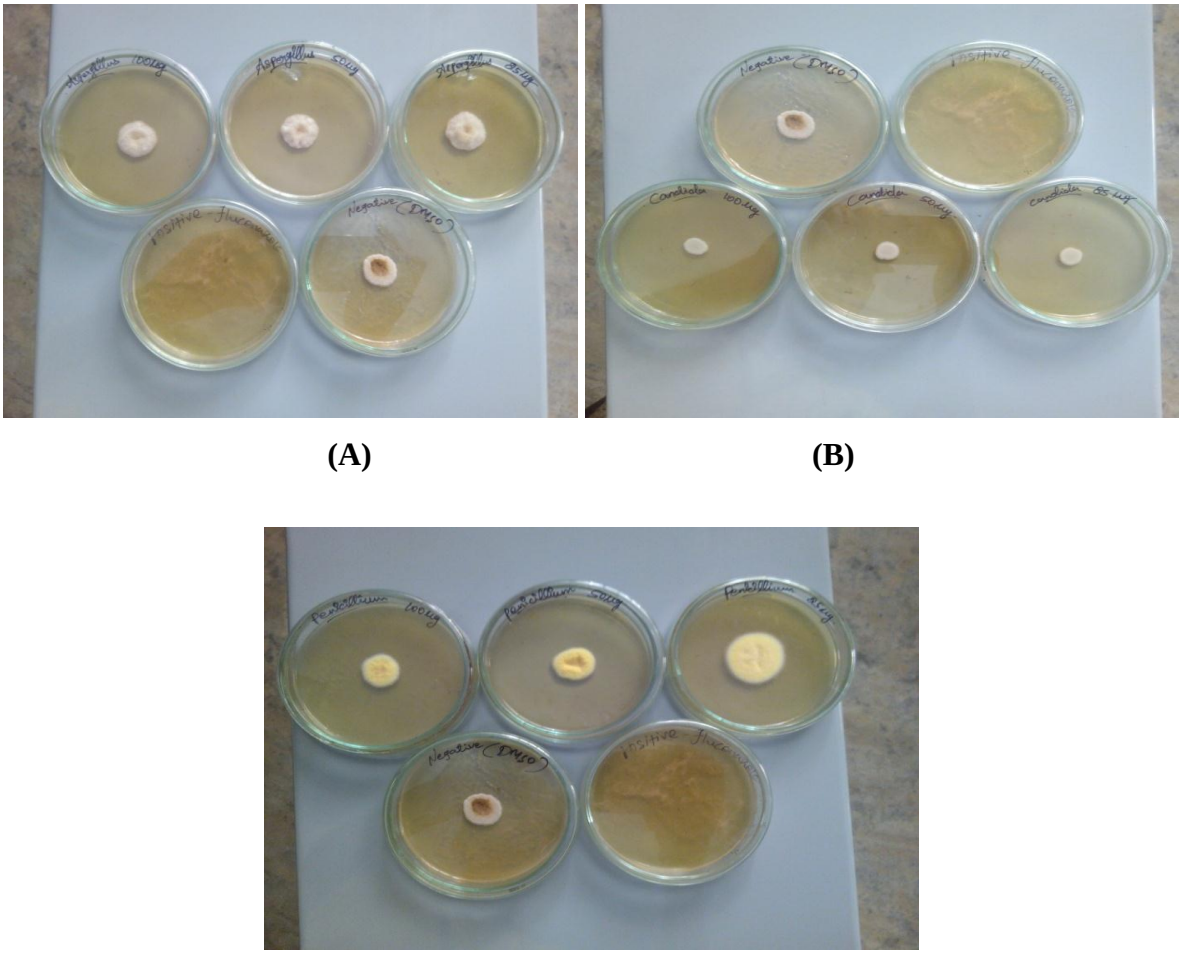
ANTIFUNGAL ACTIVITY

The percentage of growth inhibition of fungal strains namely *Aspergillus niger*, *Candida albicans* and *Penicillium chrysogenum* was noticed in the presence of copper nanoparticles at several serial dilutions.

Table.2: Antifungal activity Cu-NP at different concentration

S.NO	NAME OF THE ORGANISM	% OF INHIBITION AT DIFFERENT CONCENTRATION		
		1.0µg	**0.50µg**	**0.25µg**
1	Aspergillus niger	47.67%	31.42%	23.80%
2	Candida albicans	33.33%	26.66%	20%
3	Penicillium chrysogenum	48.57%	31.42%	17.14%

Copper Nanoparticles inhibited the growth of *Aspergillus*, *Candida* and *Penicillium* effectively in comparison with that of positive control antifungal drug fluconazole.



(A) **(B)**

(C)

Fig.7: Antifungal activity of Biosynthesized copper nanoparticles (A) *Aspergillus niger,* (B) *Candida albicans,* (C) *Penicillium chrysogenum*

ANTIBACTERIAL ACTIVITY

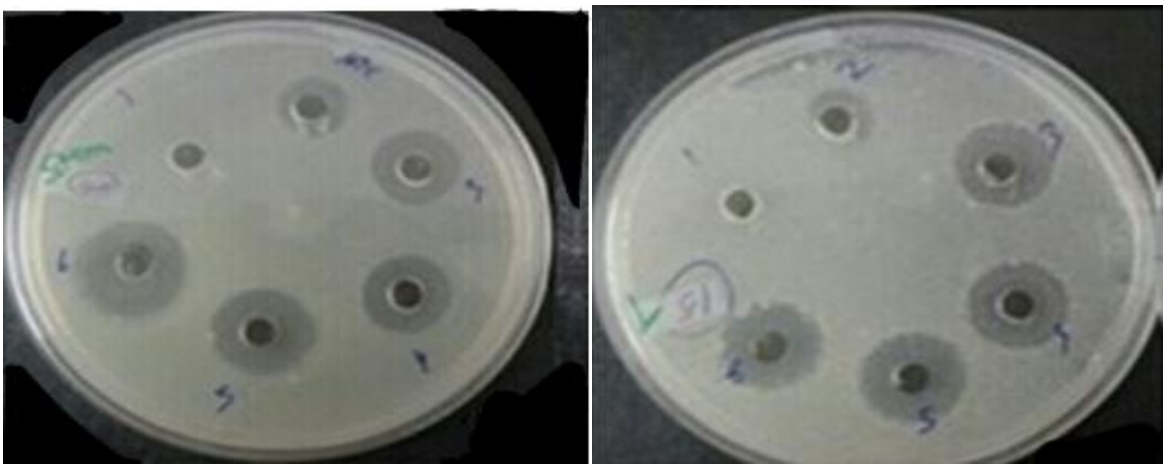
The antibacterial activity of Cu-NPs was analyzed by agar well diffusion method against the strains of E.*Coli, Klebsiella pneumoniae* and *Staphylococcus aureus*. The biosynthesized Cu-NPs showed excellent antibacterial activity against bacterial strains *E.Coli, Klebsiella pneumoniae* and *Staphylococcus aureus*. (Fig.8). The Cu-NPs synthesized using *Annona Muricata* possess discrete antibacterial activity at different concentrations ranging from 12.5-100 µg/mL.

The zone of inhibition ranges from 10 to 21mm (Table.3). The highest zone of inhibition (21mm) was, found against bacterial strain of *Staphylococcus aureus*. The MIC of Cu-NPs varies from 12.5-100 µg/ml .The antibacterial activity of Cu-NPs have depicted that as the concentration increases the antibacterial activity of Cu-NPs against bacterial strains increased

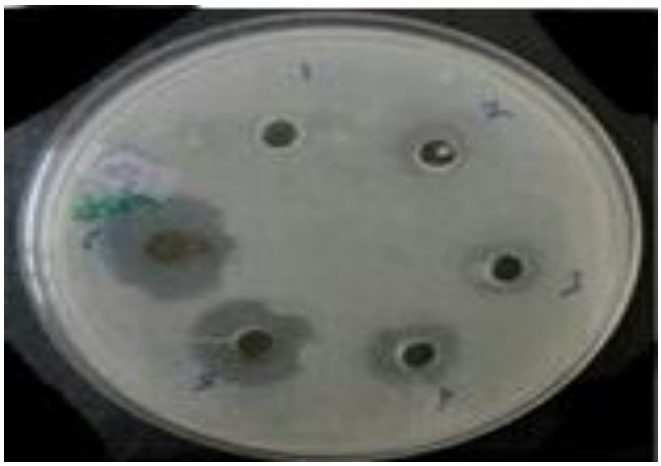
parallely. The study revealed that high antibacterial activity was found against tested strains of *Staphylococcus* at very low concentration of Cu-NPs (in µg/ml).

Table.3: Antibacterial activity of Biosynthesized Copper Nanoparticles

S.NO	NAME OF THE BACTERIA	VARIETY OF BACTERIA	ZONE OF INHIBITION (in mm)					
			12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	Ampicillin 10µg/ml
1	*E.Coli*	Gram(-)	10	11	15	16	19	13
2	*Klebsiella pneumoniae*	Gram(-)	-	13	17	17	18	12
3	*Staphylococcus aureus*	Gram(+)	10	11	13	17	21	10



(A) **(B)**



(C)

Fig.8: Antibacterial activity of Biosynthesized copper nanoparticles. (A) *E.Coli*, (B) *Klebsiella pneumoniae* & (C) *Staphylococcus aureus*

CONCLUSION

In the present study, the eco-friendly synthesis of copper nanoparticles using leaf extract of *Annona Muricata* was carried out. This method has merit over other reported methods viz easily available starting materials, cost effective, simple methodology, no usage of toxic reagents and pollution free.

In UV-Vis spectroscopy absorption peaks of Cu-NPs were observed at 279nm and 230nm. This revealed the presence of polyphenolic groups.

The XRD pattern showed diffraction angles at 22.4°, 30.6°, and 77.5°. which corresponds to the characteristic phase centered cubic of Copper lines indexed 100, 100 and 220.

SEM analysis of copper nanoparticles showed copper nanoparticles with size in the range of 40nm dynamic light scattering of nanoparticles confirmed the size distribution of copper nanoparticles within 216 nm.

Zeta potential analysis of copper nanoparticles showed a value of -13.5mV which indicated aggregation of nanoparticles due to Van Der Wall force.

FTIR spectrum of copper nanoparticles showed bands at 3256.99 cm-1 , 2919.52cm-1 , 1633.27 cm-1, 1061.29 cm-1 and 678.16 cm-1 corresponds to O-H stretching H-bonded alcohols and phenols, aldehydic C-H stretching Alkanes, -NH2 bendind, corresponds to C-O stretching alcohols and esters and bending vibrations of amines and amides respectively. FTIR spectrum of Cu nanoparticles suggested that Cu nanoparticles were surrounded by different organic molecules such as terpenoids, alcohols, ketones, aldehydes and carboxylic acid.

Antifungal activity of copper nanoparticles against *Aspergillus niger*, *Candida albicans* and *Penicillium chrysogenum* revealed that copper nanoparticles inhibited the growth with MIC value 1, 0.5 and 0.25µg/ml respectively. Which was comparable with that of Fluconazole (Positive control).

Antibacterial activity of copper nanoparticles showed zone of inhibitions in the range of 10-21nm against selected bacterial pathogens namely E.*Coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

The synthesized Copper nanoparticles were in face centered cubic shape with particle size in nanorange. This characterization has been successfully done by using SEM. Copper nanoparticles revealed to possess an effective antimicrobial property against the tested microorganisms. The increased surface area results in the enhancement of antibacterial activities of copper nanoparticles. Surface area of bacteria plays a major role while reacting with antimicrobial agents.

These synthesized copper nanoparticles can be useful in food industries, cosmetic industries, medicines and other industries. Biosynthesis of nanoparticles is a simple, fast, and biological method to synthesize copper nanoparticles. These methods provide a clean, nontoxic and eco-friendly and efficient route for the synthesis of nanoparticles with tunable particle size, at room temperature conditions without using any additive. Based on this study, some other nanoparticles may be prepared in future. Form the paint of view of nanotechnology; this is a significant advancement to synthesize copper nanoparticles.

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