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Green Synthesis, Characterization And Its Antibacterial Activity of Copper Oxide Nanoparticles Using Ocimum Sanctum Leaves And Piper Nigrum Extract

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Abstract: ***Pure copper oxide nanoparticles (CuO) were synthesized by green synthesis method at room temperature with appropriate reactants. The synthesized product was characterized by X-ray diffraction analysis (XRD), Fourier transform infrared spectroscopy (FTIR) and Scanning electron microscopy (SEM) with Energy dispersive X-ray (EDX) analysis and Transmission electron spectroscopy (TEM). XRD pattern confirmed the crystalline nature and monoclinic structure of synthesized composition. Average grain size was determined from X-ray line broadening, using the Debye–Scherrer relation. The functional groups present in the sample were identified by FTIR spectroscopy. The microstructure and nanoparticles elemental identification were done by SEM with EDX analysis. The antibacterial activity of these nanoparticles were tested against various multidrug resistance bacteria viz. both Gram-positive (B. subtilis and S. aureus) and Gram-negative (E.coli and P.aeruginosa). Further, these nanoparticles show higher antibacterial activity against B. subtilis followed by S .aureus, P. aeruginosa and E.coli.***

Keywords: Green Synthesis, Ocimum Sanctum leaves and Piper Nigrum Extract, CuO NPs, Antibacterial Activity.

I. INTRODUCTION

Copper oxide nanoparticles have attracted significant attention because of their wide range of applications such as high-Tc superconductors[1, 2&3],sensors[4], catalytic[5,6,&7], optical[8], electrical[9],giant magnet resistance material, solar energy transformation and preparation of organic–inorganic nanostructure composites [10&11]. CuO NPs is a p-type semiconductor with the band gap of~1.7 eV[12]. Further it can be used as anti-bacterial agent when incorporated in coatings, plastics textiles, etc[13]. Copper and copper-based compounds are efficient biocidal properties, which are generally used in pesticide formulations[14] and several health related applications. Different methods available to prepare CuO-NPs namely sol–gel technique[15], solution combustion methods[16], electrochemical methods[17], precipitation[18], microwave irradiations[19], solid-state reaction method[20], thermal decomposition of precursor etc[21].

These methods involve high temperature, high pressure and hazardous chemicals, and some toxic chemicals absorbed on the surface of nanoparticles may cause adverse medical effects[22].Green synthesis of nanoparticles has several advantages over chemical and physical synthesis, method using leaves extract. In addition, the plant-mediated synthesis is a rapid, flexible, and suitable process for large-scale production of nanoparticles[23]. Among nanoparticles copper oxide nanoparticles have been used enormously due to their potent antibacterial activity.

In recent, green synthesis of Copper oxide nanoparticles was achieved by using microorganisms[24], plant extract[25], seed[26]and bark[27]. Ocimum sanctum an Indian origin is considered as Holy basil in India. Ocimum sanctum belongs to the family Lamiaceae, which is well known for its medical use. In Ayurveda, the Indian system of medicine since ancient times, Ocimum sanctum attributes several medicinal properties. Ocimum sanctum (local name Tulasi) is a traditional medicinal plant of India has a source of bio-reduction and stabilizers. The constituent of Ocimum sanctum are alkaloids, glycosides, tannins, saponins and aromatic compounds. It is used in the treatment of headaches, coughs, diarrhea, constipation, worms and kidney malfunctions[28]. Hence it is also termed as the Queen of Herbs. Ocimum sanctum remains as an active area of scientific research for both human nutritional needs and therapeutic applications. Recently Ocimum sanctum leaf extracts have been used in the synthesis of metal oxide nanoparticles[29].

In the group of medicinal plants, the *Piper nigrum* possess excellent medicinal properties due to the presence of enormous phytochemicals. The piperine is an alkaloid, majorly found in *Piper nigrum*, which belongs to the Piperaceae family that is

massively cultivated at India and Sri Lanka[30&31]. Due to the presence of enormous quantity of phytochemicals, the powder of Piper *nigrum* (black pepper) are taken under consideration for the synthesis of copper oxide nanoparticles.Pipernigrum is native to south India and is extensively cultivated there and elsewhere in tropical regions. The pepper plant is a perennial woody vine growing up to 4 metres (13 ft) in height on supporting trees, poles, or trellises. It is a spreading vine, rooting readily where trailing stems touch the ground. Black pepper (Piper nigrum) is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spiceand seasoning. When dried, the fruit is known as a peppercorn. When fresh and absolutely mature, it is approximately 5 millimetres (0.20 inch) in diameter, dark red, and, like all drupes, contains a single seed. Peppercorns, and also the ground pepper derived from them,is also repesented merely as pepper, or more precisely as black pepper (cooked and dried unripe fruit).

The present study attempts to fill the knowledge gap by investigating the synthesis, stability and antibacterial ability of CuO-NPs synthesized by Ocimum sanctum leaf and piper nigrum extract. O.sanctum leavesand P.nigrum extract solution was used as a reducing and capping reagent for the CuO-NPs synthesis and distilled water served as the reaction medium. The reaction conditions on the synthesis of CuO-NPs were studied. The obtained particles were analyzed by X-ray diffraction(XRD), Fourier transform infrared (FT-IR) spectroscopy, Scanning electron spectroscopy (SEM), Energy dispersive spectroscopy (EDS), Transmission electron microscopy (TEM) analyzer to understand the morphology and capping of CuO NPs. In addition, the antibacterial effect of copper oxide nanoparticle is examined equally effective against plant pathogens such as Gram +ve (B. subtilis and S. aureus) Gram –ve (P. aeruginosa and E.coli.) Bacteria using Kirby Bauer disk diffusion method respectively[32&33].

II. MATERIALS AND METHODS

A. *Materials*

All reagentswere of analytical grade with 99% purity. Copper nitrate [Cu $(NO_3)_2.3H_2O$] was bought from Hi-media laboratories, Mumbai, India. All chemicals and reagents were used as acquired without in addition purification. Deionized distilled water was used in all experimental work.Ethanol was used for the washing purpose.

B. *Collection Of Leaves*

Ocimum sanctum leaves were collected from agricultural lands of rural villages and the Piper *nigrum* were brought from the departmental store in Chidambaram, Cuddalore district in Tamilnadu .The selected leaves were washed in running tap water several times and then washed with distilled water 2-3 times to remove dust particles and then dried at room temperature for removal of residual moisture.

C. *Preparation Of Ocimum Sanctum Leaves And Piper Nigrum Leaves Extract*

About 20g of fresh healthy leaves of *Ocimum sanctum* leaves and 5g of *Pipernigrum* cut into fine pieces with a sterile knife. About 25 g of chopped leaves was weighed and taken in a beaker and 250 ml Erlenmeyer conical flask and 50ml of double deionized water was added to it. It was boiled for 30 min at 80°C. By this time aqueous part turns light brown colour. After cooling, the extract was filtered through Whattman no.1 filter paper and stored at 4°C for further usage. It was used as the reducing and stabilizing agent.

D. *Synthesis Of Cuo Nps*

In order to synthesize CuO NPs, 2ml of leaves extract was added to 25ml of 1mm copper nitrate trihydrate [Cu $(NO_3)_2.3H_2O$] was added into 25 ml of double distilled water and boiled 70-80°C by using magnetic stirrer. Color change of reaction mixture was observed from deep blue to brownish black precipitate was formed and was washed repeatedly with deionized water followed by ethanol to remove the impurities. The precipitate was transferred to a ceramic crucible followed by heating in furnace at 300°C for 3 hours. The obtained product of the black precipitate is grinded using pestle and mortar. The black colored CuO-NPs were stored in properly labeled air tight container for further characterization.

E. *Characterization*

The FT-IR spectra analysis was on a Jasso FT-IR/4100 spectrophotometer with $4cm^{-1}$ resolution in the range of 4000–400 cm^{-1}. XRD patterns of the products were recorded on a Shimadzu XRD-6000 X-ray diffractometer at a scanning rate of $0.05°s^{-1}$ with a 2θ range from 10°to 80°, with high-intensity Cu Kα radiation (λ = 0.154178 nm). Scanning electron microscopes and energy dispersive X-ray analyses were obtained by JEOL JSM- 6700 SEM (operating at 10 kV) and these nanoparticles were recorded using a TEM operating at an acceleration voltage of 15kV (JEM-1200EX, JEOL Ltd., Japan). Further, antibacterial activity against various bacterial pathogens viz. both Gram-positive (*B. subtilis*and *S. aureus*) and Gram negative (*P. aeruginosa* and *E.coli.)* carried out.

F. Determination of antibacterial activity of copper oxide nanoparticles by the kirby bauer disk diffusion method:

Antibacterial activities of the green synthesized copper oxide nanoparticles were investigated against two Gram- positive (Bacillus subtilis and Staphylococcus aureus) and two Gram-negative (Escherichia coli and Pseudomonas aeruginosa) bacteria was obtained from National collecting Industrial Micro organisam (NCIM), Biochemical Sciences Division, National Chemistry laboratory, Pune.

III. RESULT AND DISCUSSION

A. *Structural analysis*

The XRD technique was used to determine and confirm the crystal structure of the nanoparticles. Crystal structure and phase analysis of copper oxide nanoparticles synthesized usin Leaves extract have been recorded in the diffraction angle range 20–80°. The XRD analysis showed a series of diffraction peaks at 2θ of 32.5°, 35.54°, 38.67°, 48.81°, 53.8° , 58.28°, 61.52°,66.2°, 68.04°, 72.7° and 75.5° which were assigned to (110), $(\bar{1}11)$, (111), $(\bar{2}02)$, (020), (202),$(\bar{1}13)$,$(\bar{3}11)$, (220),(311) and $(\bar{2}22)$ planes respectively. The XRD spectrum clearly suggested the crystalline nature of the CuO NPs synthesized using leaf extract of *O.sanctum* and *P.nigrum*. The peak positions exhibited the monoclinic structure of CuO NPs which was confirmed by JCPDS card no. 895899 (Fig. 1) . The peak widths and intensities clearly indicate that copper oxide was highly crystalline in nature. The average crystallite size of CuO nanoparticles was calculated using Scherrer formula, D=0.9 λ/β cos θ, where λ is the wavelength of X-ray radiation, β is the full width at half maximum (FWHM) of the peaks at the diffracting angle θ. It was found to be about 30 nm indicating its nano crystalline nature. The lattice parameters a, b, c was calculated by applying the following equation:

$$\frac{1}{d^2} = \frac{1}{\sin^2\beta}\left(\frac{h^2}{a^2} + \frac{k^2\sin^2\beta}{b^2} + \frac{l^2}{c^2} - \frac{2hl\cos\beta}{ac}\right) \qquad (1)$$

The extracted diffraction peaks could be indexed to monoclinic phase of CuO (space group C2/c) and the lattice contants calculated for the CuONPs which is in close agreement with the standard data (lattice parameters a=4.682A°, b=3.424A°, and c=5.127A°, β=99.420 and cell volume V=81.08A^3), with product indicated the purity of the sample. Increasing *Ocimium sanctum* and *Piper nigrum* leaf extract concentration upto 6ml, giving rise to small distortion in the lattice constant and thus degrades the crystallinity, in Fig.2. There is a slight shift in the XRD peak position towards lower angles with increase in *Ocimum sanctum and Piper nigrum* leaf extractconcentration, resulting in change in the lattice constant. Structural parameters of CuO nanoparticles for different leaf concentration are shown in Table 1. Lattice constants increase with increase in the *Ocimum sanctum and Piper nigrum* leaf extract concentration. By utilizing the slope of the Williamson- Hall plot[34] on the basis of the powder diffraction peak broadening, D crystalline size of CuO nanoparticles was evaluated using Debye – Scherrer formula, α lattice constant and width of a diffraction peak at half maximum intensity (a) into the following equation[35].

$$\delta = 15\ \beta\ \cos\theta / 4\ \alpha\ D$$

Obtained crystallite size, strain(ϵ) and dislocation density of both samples were calculated by the following reference[36] are shown in Table 2.

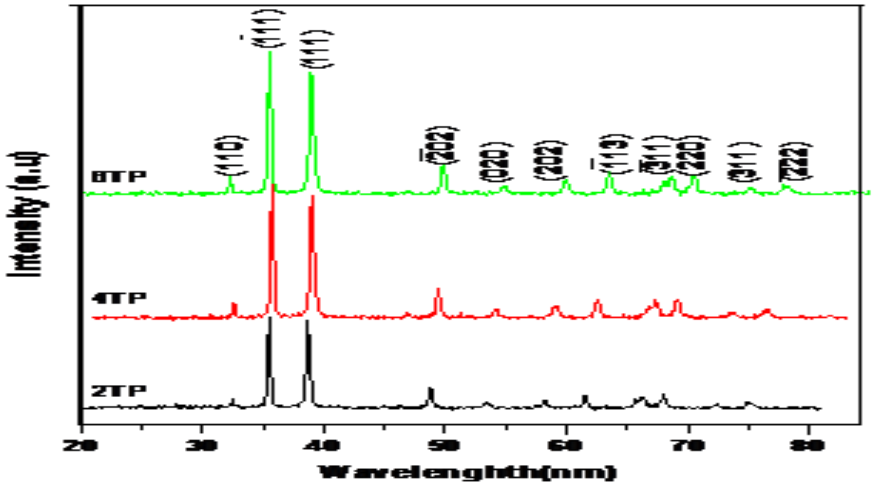

Figure 1.The XRD pattern of the CuO NPs

Table.1. Measured Lattice Parameters and Cell Volume of CuO Nps

Sample Code	Unit Cell Parameter (nm)		Cell Volume (v^3)	
	Standard	Measured	Standard	Measured
2TP	a = 4.682	a = 4.723	81.28	81.55
	b = 3.424	b = 3.431		
	c = 5.116	c = 5.104		
4TP	a = 4.682	a = 4.729	81.28	81.90
	b = 3.424	b = 3.428		
	c = 5.116	c = 5.124		
6TP	a = 4.682	a = 4.716	81.28	81.43
	b = 3.424	b = 3.422		
	c = 5.116	c = 5.118		

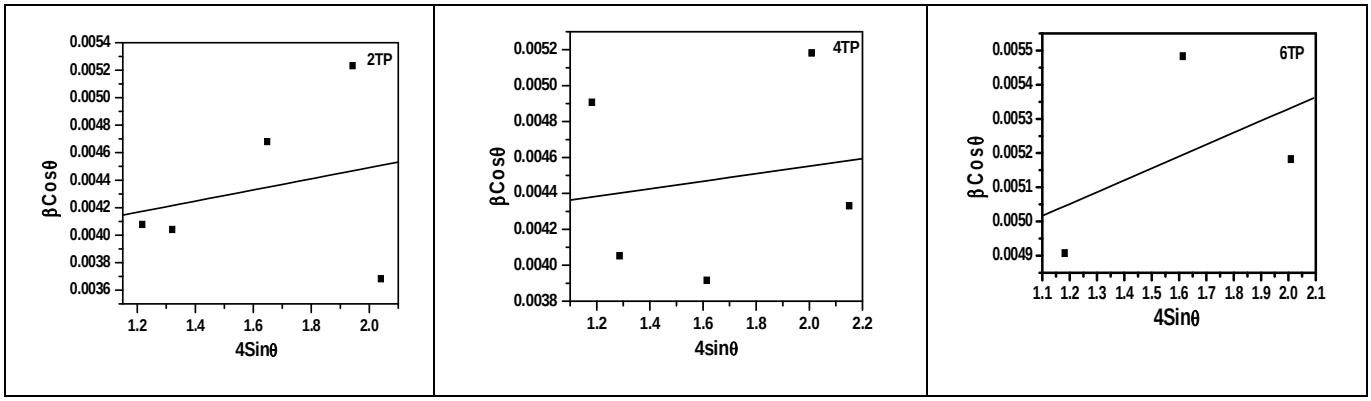

Figure 2. W-H Plot Of CuONPs.

Table .2.Structural parameters of CuO nanoparticles.

Sample Name	Particle size (nm)		Strain	Dislocation Density (m^{-2})
	W-H Method	Scherrer Method		
2TP	37.673	32.417	0.00041	9.96738E14
4TP	33.568	31.324	0.00021	1.05574E15
6TP	29.943	26.763	0.00035	1.40474E15

The dislocation density (δ) of the CuO NPs is shown in Table 2. Increasing grain boundary in the sample caused by a decrease in the grain size led to increase the dislocation density as well as the hardness of materials owing to the preventing the movement of a dislocation by the others at grain boundaries. On the other hand, the crystals have grown completely and have enhanced their size by increasing the concentration of leaf extract.

B. Ft-Ir Analysis

The FT-IR spectra were recorded using FT-IR spectrometer. A known amount of sample was ground with KBr and the pellet form of the samples was analyzed with FT-IR instrument. FT-IR measurement was carried out to identify the possible molecules responsible for capping and reducing agent for the copper oxide nanoparticles synthesized using O.sanctum leaves and piper nigrum extract stabilizer[37]. FT-IR spectra were recorded in the range between 4000 and 400 cm^{-1}. Fig.3. illustrates the FT-IR spectra of green synthesized CuO nanoparticles. The FTIR spectrum of CuO nanoparticles in Ocimum sanctum and Piper nigrum extract exhibit an absorption band in 3748 cm^{-1} for 2TP. It is shifted to higher frequency region in 3772cm-^{1}, 3794cm^{-1} for 4TP, 6TP due to stretching vibration of OH molecules[38]. The band around at 2328 cm^{-1} for 2TP is shifted to 2362cm^{-1}, 2374cm^{-1} in 4TP, 6TP, assigned to carboxylic group (COO^{-}) vibration. The presence of a peak at 1515 cm^{-1} and 1314cm^{-1} for 2TP and it is shifted to 1600cm^{-1},1612cm^{-1},1420cm^{-1},1445cm^{-1} in 4TP,6TP corresponding to C=C stretching and C=N stretching respectively[39]. The high

intensity absorption bands indicate around 549 cm^{-1}, 561Cm^{-1}, and 573 cm^{-1} are due to the Cu - O stretching in the monoclinic structure of CuONPs[40,41&42]. FTIR spectrum of CuO nanoparticles suggested by different organic molecules such as alcohols, ketones, aldehydes and carboxylic acid[43]. From the analysis of FTIR studies we confirmed that the carbonyl group from the amino acid residues and proteins has strongly ability to bind metal indicating that the proteins could possibly from the metal nanoparticles(i.e. capping of copper oxide nanoparticles) to prevent agglomeration and thereby stabilize the medium. In 4TP, 6TP all the peaks showed a blue shift to some extent. This means that the bands move along the direction towards higher frequency from the standard values. The small peak shift in the vibrational modes is associated with the corresponding change in the surface area of the prepared CuO NPs. The shifting in these bands is clearly indicating that the coordination of hydroxyl, carbonyl of the amino acids in the protein of Ocimum sanctum and Piper nigrum leaf extract with CuO NPs play a major role on dispersing, stabilizing and capping of CuO NPs[44&45&46].

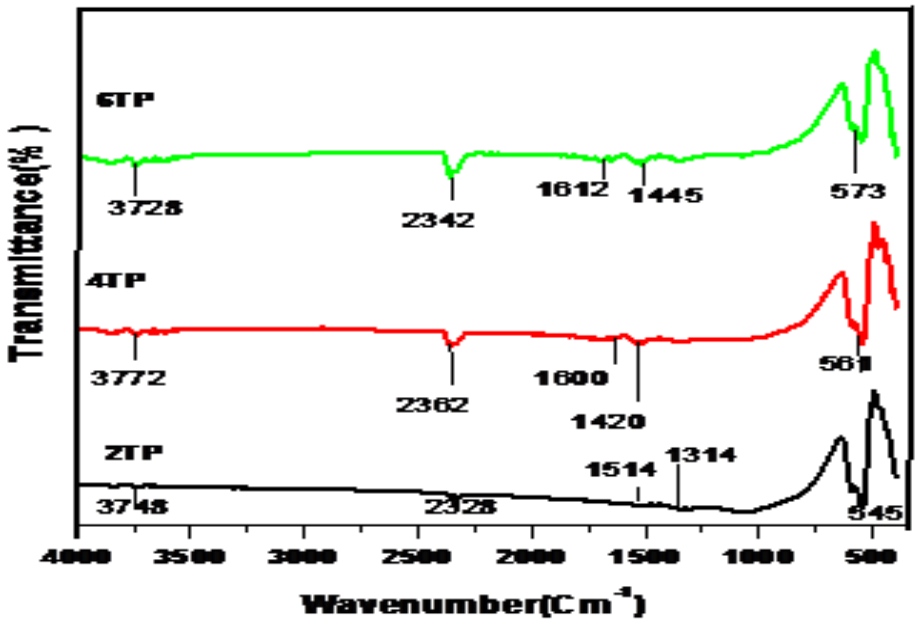

Figure 3.FTIR Pattern Of CuO Nanoparticles.

C. Scanning Electron Microscopy (SEM) Analysis

Morphology of synthesized CuONPs and bio functionalized form was characterized by SEM technique at different magnification levels. SEM images were recorded using JEOL Model JSM - 6390LV SEM. In general, in this analysis, the samples were placed in an evacuated chamber and scanned in a controlled pattern by an electron beam. Since the interaction of the electron beam with the samples produced a variety of physical phenomenon that detected and used to form images then provide information about the specimens. Fig.4. SEM image exhibited that the green synthesized copper oxide nanoparticles have slight agglomeration due to nanoparticles oxidation[47&48&49].

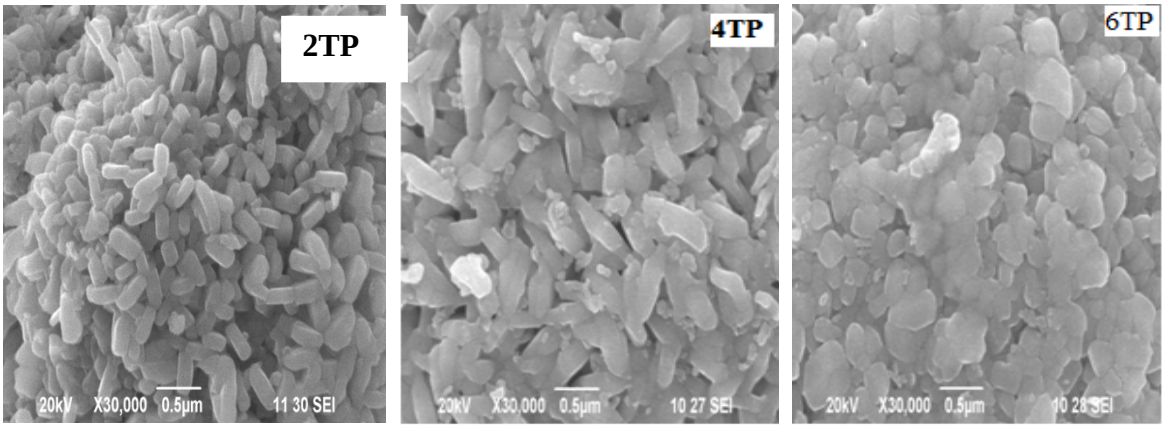

Figure 4.SEM IAGE OF CuO NANOPARTICLES

D. Energy Dispersive X-Ray (EDX) Analysis

Figure.5. showed the EDX analysis of CuO-NPs annealed at 400˚C. The EDX analysis was carried out CuO NPs at 10 keV. Results revealed the presence of copper (Cu) and oxygen (O) elements in CuO-NPs.The weight percent of copper and oxide calculated from EDX analysis were O: 22.04 weight % (0.525 keV) and Cu: 77.96 weight % (1.204 keV), respectively. There were no other elemental impurities in the EDX spectra. This result confirmed the formation of pure CuO-NPs[50].

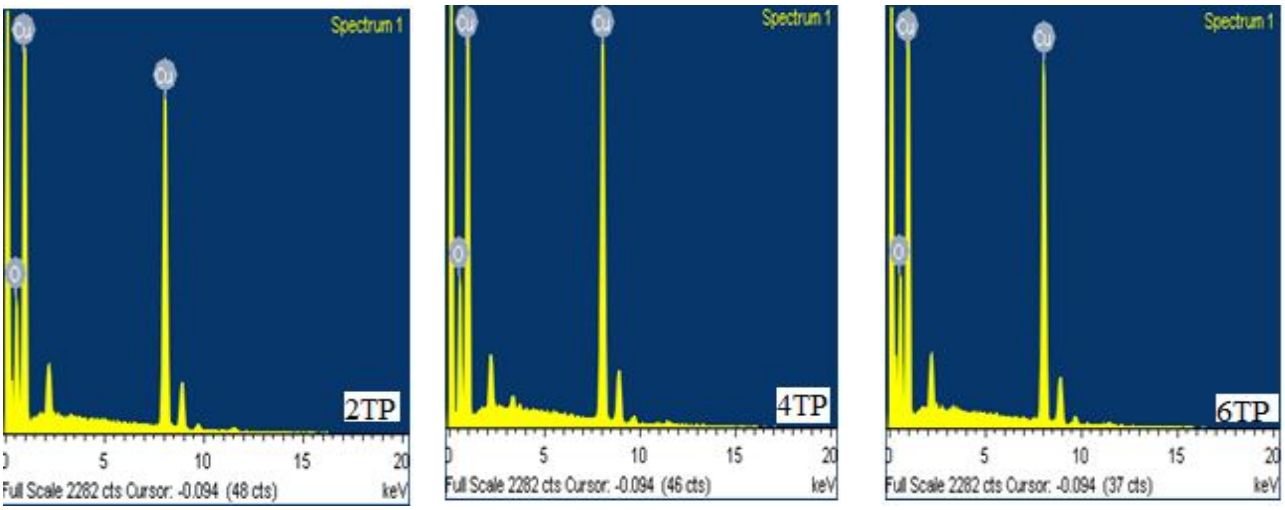

Figure5.EDAX SPECTRA OF CuO NANOPARTICLES

E. Transmission Electron Microscopy Analysis (Tem)

TEM has been employed to characterize the size, shape and morphology of synthesized copper oxide nanoparticles derived from O. sanctum and P. nigrum respectively, and in combination. The CuO nanoparticles synthesized by the present method are smaller in size. Figure 6a. shows the TEM image of copper oxide nanoparticles synthesized using copper nitrate stabilized by O.sanctum leaves and P.nigrum extract. Figure 6b shows the crystallinity of the copper oxide nanoparticles, which are by selected area emission diffraction (SAED). SAED pattern of the synthesized CuO NPs exhibits the dotted concentric rings of monoclinic CuO-NPs. The spots were formed by the diffraction of transmitted electrons through the monoclinic CuO NPs in different orientations.The diffraction rings correspond with planes (110),(111) ,(200) and ($\bar{1}$12) are correlated for CuO NPs. The well separated lattice fringes while the measured d-spacing of 0.273 nm was found,which is in good correlation with the calculated d-spacing of the (110) plane (ie) 0.27nm[51].

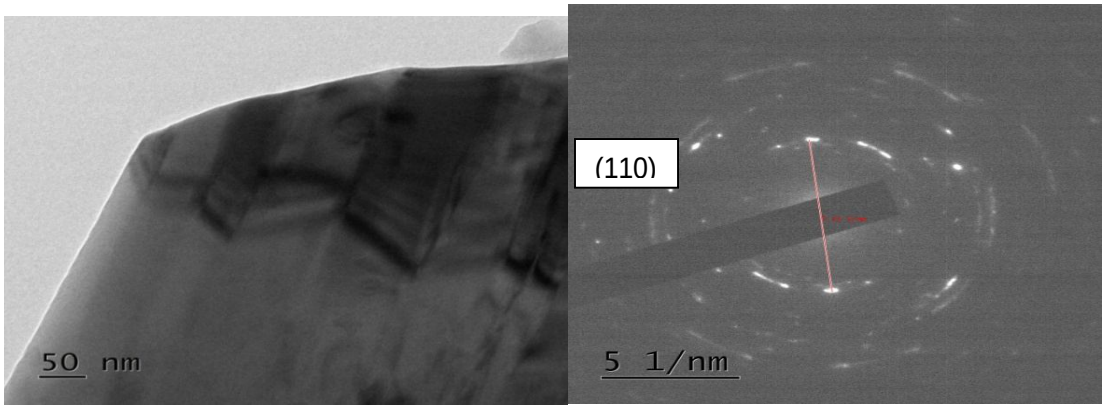

Fig. 6a. TEM Image of CuONPs

Fig. 6b. SAED Pattern of CuONPs

F. Antibacterial Activity

Antibacterial activity can be attributed to disruption of cell membrane due to the release of copper ions from CuO NPs, which attach to negatively charged bacterial cell wall and rupture it, thereby causing protein denaturation and cell death. Once entered into

bacterial cell, it may bind to deoxyribonucleic acid molecules and get involved in cross-linking of nucleic acid strands, forming a disorganized helical structure. The studies are in conformity with the Green synthesis of copper oxide nanoparticles using natural reducer and stabilizer in an evaluation of Antibacterial activity[52,53]. In addition, copper ion uptake by the bacterial cells also interrupts important biochemical processes[54].

The bacterial properties of green synthesized CuO NPs against Gram +ve (B. subtilis and S. aureus) Gram –ve (P. aeruginosa and E.coli.) bacterial pathogens. Concentrations ranging from 10 to100 µg/mL were used to evaluate the minimum inhibitory concentration of CuO NPs. MIC was observed at 20 µg/mL, with zone of inhibition at 26mm,19mm,32mm and 38mm for B. subtilis , S. aureus,P. aeruginosa and E.coli respectively. An increase in the size of inhibition zone was noticed for all the bacteria tested with increasing concentration of CuO NPs for 50 µg/mL and 100 µg/mL (bar diagramFig.4). The sensitivity of B. subtilis is higher at high concentrations of CuO NPs (50–100 µg/mL) with maximum zone of inhibition,then compared to other bacterial pathogens. However, CuO NPs with 100 µg/mL concentrations appears to be effective as there was no bacterial growth, with zones of inhibition for S. aureus, P. aeruginosa and E.coli being 12mm, 15mm and 16 mm, respectively (Fig. 5). Gentamicin (standard antibiotic) was used as positive control. For comparison with standard antibiotic, we have tested zone of inhibition of Gentamicin with 100 µg/mL concentration showing 19 mm for all the selected bacterial pathogens. Dissolving solution (DMSO) extract was used as negative control which does not have any zone of inhibition. CuO NPs showed excellent antibacterial activity against all the four bacterial pathogens even at lower concentrations, i.e. Above 50 µg/mL (Fig. 6).However, the dose of 100 µg/mL was found to be effective for inhibiting the growth. Antibacterial activity of CuO NPs extract was considered to be good if its MIC was less than 12.5 µg/ml, moderate if MIC was from 150.0 to 500.0 µg/ml and poor over 500 µg/ml.

TABLE:3.ZONE OF INHIBITION TABLE CuO NANOPARTICLES

Name of the Bacterial Strains	Name of the Sample	50µl	100µl	Gentamicin	MIC (µl/disc)
Bacillus Subtilis	2TP	10.2±0.29	20.3±0.56	19.2	26.7± 0.35
	4TP	9.5± 0.54	13.2±0.43	20.7	
	6TP	12.3±0.40	14.7±0.71	21.5	
Staphylococcus aureus	2TP	9.8±0.75	12.4±0.48	19.8	19.8± 0.33
	4TP	8.7±0.81	10.5±0.87	20.3	
	6TP	9.6±0.67	11.7±0.39	21.8	
Eschericila coli	2TP	8.2±0.47	16.2±0.27	19.1	32.5± 0.52
	4TP	7.8±0.39	17.5±0.59	20.9	
	6TP	10.9±0.13	12.8±0.67	21.4	
Pseudomonas aeruginosa	2TP	8.6±0.27	15.5±0.13	19.3	38.3± 0.43
	4TP	7.3±0.63	11.3±0.36	20.6	
	6TP	8.7±0.29	12.7±0.49	21.8	

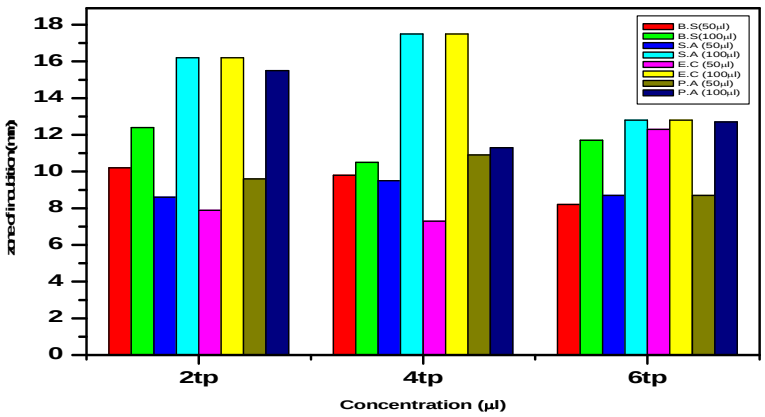

Figure 7.Zone Of Inhibition Of Cuo Nanoparticles

IV. CONCLUSION

In conclusion, the above results suggest the Ocimum sanctum leaves and piper nigrum extract can synthesize copper oxide nanoparticles in economical and eco-friendly manner. The XRD analysis confirms that copper oxide nanoparticles are crystalline in nature with monoclinic structure and the average crystalline size was found to be 30 nm. Copper oxide nanoparticles were thermally stable and can act as good antibacterial agent. The synthesized copper oxide nanoparticles and bio-functionalized nanoparticles morphology and size were investigated by SEM and TEM analysis. The antibacterial activity was also investigated against two gram-positive and two gram- negative bacteria. From the inhibition zone results, CuO NPs were showed better inhibition activity against B.subtilis bacteria compare to the other bacterial strains. Thus our findings report the bio-functionalized copper oxide nanoparticles synthesized from the Ocimum sanctum and Piper nigrum leaf extract by green method are shown promise results in the review of pharmaceutical and therapeutic applications make this method potentially use for the large-scale synthesis of other inorganic nanomaterials.

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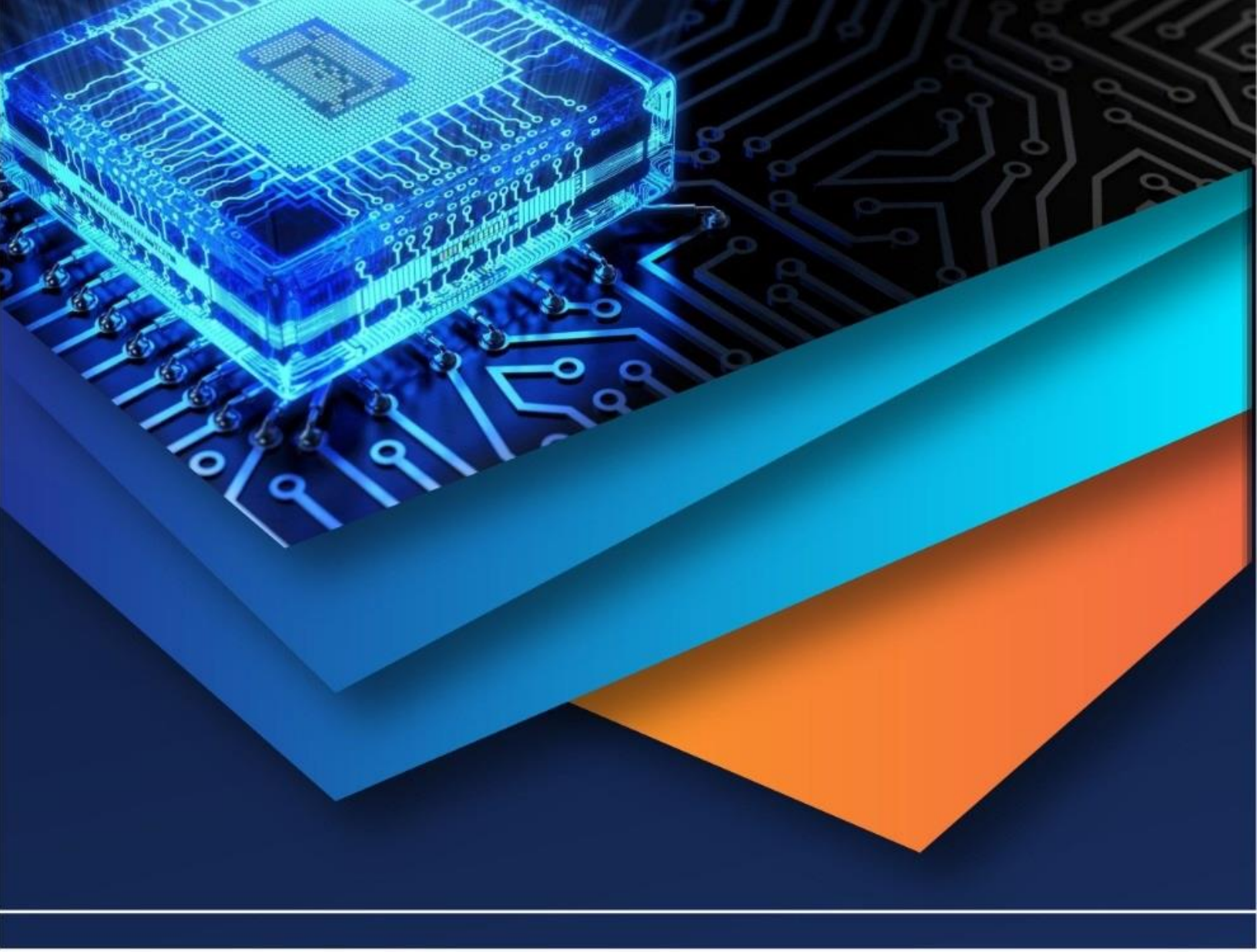

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