PLANT MEDIATED GREEN SYNTHESIS OF COPPER OXIDE NANOPARTICLES AND THEIR ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY

S.Manikandan[a], M.Senthilkumar[b*],B. Ravindran[c] and K. Ramesh[d]

[a]*Ph.D Scholar, Department of Chemistry, Government Arts College (Autonomous) (Affiliated to Bharathidasan University), Kumbakonam- 612002, Tamil Nadu, India*

[b]*Assistant Professor, Department of Chemistry, Government Arts College (Autonomous), Kumbakonam- 612002, Tamil Nadu, India*

[c]*Assistant Professor, Department of Physics, Thiru. Vi. Ka. Government Arts College, Thiruvarur-610003, Tamil Nadu, India*

[d]*Assistant Professor, Department of Chemistry, Poombukar College (Autonomous), Melaiyur-609107, Tamil Nadu, India*

Abstract

This paper presents a green synthesis of plant modified copper oxide nanoparticles (CuO NPs) using *Justicia adhatoda* L. (*J. adhatoda*) aqueous leaves extract. The nano particles of CuO were synthesized by using $CuCl_2.2H_2O$ and *Justicia adhatoda* L. aqueous leaves extract of various concentrations (1% - 5%) with simultaneous addition of NaOH at 80^oC. The synthesized CuO NPs were studied by XRD (X-ray diffraction)), Ultra violet- Diffuse reflectance spectroscopy (UV-DRS), Fourier transform infrared spectroscopy (FT-IR). The morphology of sample was studied by Scanning electron microscopy (SEM) and High-resolution transmission electron microscopy (HR-TEM). The antibacterial activity of synthesized CuO NPs was executed against gram-positive bacteria of *Staphylococcus aureus* (strain MTCC 6908), *Bacillus subtilis* (strain MTCC 0441) and gram-negative bacteria of *Escherichia coli* (strain MTCC 1557) and *Klebsiella aerogenes* (strain MTCC 0039). The antifungal activity of synthesized CuO NPs was studied against the funguses of *Aspergillus fumigatus* (strain MTCC 0343),*Aspergillus niger* (strain MTCC 1344),*Candida albicans* (strain MTCC 0227)and*Penicillium chrysogenum* (strain MTCC 0947). The XRD pattern reveals the formation of single phasic CuO with monoclinic structure up to 2% concentration of plant extract. A peak at 370 nm in UV-DRS spectra shows the evolution of CuO NPs and a band gap of 1.47eV is observed. Peaks were obtained in FT-IR confirms that the CuO NPs is formed from bioactive compounds containing in plant extract. SEM and HR-TEM images depicts that CuO NPs are spherical morphology and the mean value of grain size are differing

from 20 to 40 nm. Antibacterial activity finds the maximum zone of inhibition (27.2 ±0.9 mm) with *E. coli* in 250µg/L. The synthesized CuO NPs also shows good antifungal activity and finds the maximum zone of inhibition with *P.chrysogenum* is 17.7±0.6 mm in 250µg/L. Thus, this process can be utilized to synthesize stable CuO NPs via rapid, eco-friendly and cost-effective technique. CuO NPs is observed to be a promising candidate for better antibacterial and antifungal agent.

Keywords: CuO NPs; Plant mediated Synthesis; Justicia adhatoda L. leaves extract; Antibacterial activity; Antifungal activity.

* Corresponding author. Tel.: +91-948 624 7438

*E-mail address:*mskumargpm2003@gmail.com

1. Introduction

Now a day's metal oxides play a significant role in many industrial sectors. In which copper oxide (CuO) have been playing a vital role due to the characteristics of catalytic [1], electric [2], photocatalytic [3], optical [4], antioxidant [5], anticancer [6] and antibacterial properties [7]. In recent time metal oxide nanoparticles are finding enhanced applications than the bulk materials. Therefore, researchers are showing keen interest in the synthesis of metal oxide in nano scale. There are many bottom-up techniques such as sol–gel technique [8], sonochemical [9], alkoxide based route [10], electro chemical methods [11], precipitation-pyrolysis [12], microwave irradiations [13], solid-state reaction method [14], thermal decomposition of precursor [15] and phytogenic synthetic route are reported to synthesize metal oxide nanoparticles. Among these, phytogenic synthetic route finds considerable attention due to eco-friendly, non-toxic, and cost effective. The stabilizing agent used in conventional methods leads to pollute the environment. It is evident that the constituents in plant containing phytochemicals such as poly phenolic play an important role of stabilizing and reducing agent for the synthesis of metal oxide nanoparticles [16]. On this basis, many attempts have been made to synthesize CuO NPs using plant such as *Malva sylvestris* [17], *Cassia auriculata* [18], *Carica papaya* [19] and *Indigofera tinetoria* [20]. Recently copper compounds attracted more interest due to its high antibacterial and antifungal activity [21]. Several nanoparticles of metallic compounds have been reported so far such as copper, silver and gold which exhibit strong antimicrobial activity. Among these compounds, copper attracts much attention to researchers due to its cheaper cost and good antimicrobial activity [22]. In 1761, copper was used in the form of a weak copper sulphate solution soaked with seed grains to prevent seed-borne fungi in agriculture work for the first time. As a metallic fungicide, the compounds of copper play vital role in agricultural application practices [23].

A mixture of lime, water and copper sulphate known as Bordeaux mixture and Burgundy mixture (composition of sodium carbonate and copper sulphate) were used as a prospective fungicide in France and United states in 1880's for the fight against mildew on grapes. In the era of nineteenth century, scientists made an observation that the cholera made no effects on copper workers from USA. Hence from 20th century usage of Cu compounds became widespread [24]. It is unfortunate that the compounds of copper may became toxic for fish and other organisms [25]. Biosafety study on Cu NPs, CuO and $CuSO_4$ showed that Cu NPs are less toxic than other copper compounds [26]. But the synthesis of Cu NPs is challenging due to their high tendency to oxidation. Oxidation of copper results in the formation of Cu_2O and CuO converts into Cu^{2+} during the process of preparation and storage [27]. Comparing CuO and $CuSO_4$, CuO is less toxic. Another study divulged that the CuO NPs are effective antimicrobial properties than the bulk form of CuO and it is also stable than Cu NPs [28]. The present studyreports the synthesis of CuO NPs using aqueous extract of *J. adhatoda* leaves. *J. adhatoda* commonly is known as Malabar mut in English and Adhatoda in Tamil. It is a tropical shrub with lance-shaped leaves of 10-15 cm by its length. It belongs to the family of *Acanthaceae* and genus of *Justicia*. *J. adhatoda* is heritage used to treat cough, cold, tuberculosis and asthma [29]. Physico-chemical screening of *J. adhatoda* leaves was reported elsewhere [30-31]. From the literature study, it is observed that *J. adhatoda* (whole plant parts) contains rich in alkaloids, tannin, saponin, phenolics and flavonoids. CuO NPs and with carbon composites using *J. adhatoda* leaves were synthesized by P.G. Bhavyasree *et al*., K. S. Dayana *et al*., and Md. J. Islam *et al*., [32-34]. $CuSO_4$ was used as predecessor for synthesis of CuO NPs and studied antibacterial property for several bacterial species.

The current investigation explores the synthesis of CuO NPs using *Justicia adhatoda* L. leaves aqueous extract. It observes the probable utilization of CuO NPs as a antibacterial and antifungal agent against bacterial species of *Bacillus subtilis,Staphylococcus aureus,Escherichia coli* and*Klebsiella aerogenes* and fungal species of *Aspergillus fumigatus,Aspergillus niger,Candida albicans* and *Penicillium chrysogenum* species.

2. Experimental methods

2.1. Preparation of plant extract

J. adhatoda leaves plugged freshly and washed several times with tap water and with deionized water were collected. The collected leaves were dried with air at room temperature for couple of weeks.About 5g of air-dried leaves were weighed to prepare 5% stock solution. The weighed air-dried leaves of *J. adhatoda* were cleaned again in distilled water and the same was chopped into very small pieces. The leaves were made as a fine paste using pestle

and mortar and then 250mL distilled water was added. It was heated to 60^0C with simultaneously stirring for 30 min using magnetic stirrer. The colloidal solution was filtered through filter paper of Whatman No.1 and then it was kept in freezer for future studies.

2.2. Synthesis of CuO nanoparticles

For the formation of copper oxide nanoparticles analytical grade of copper chloride dihydrate ($CuCl_2.2H_2O$, 98.5% purity, Merck), sodium hydroxide (NaOH, ≥97.0% purity, Merck) and ethanol (99.9%) were used. From the stock solution (5%) of plant extract, 1%, 2%, 3% and 4% respectively were prepared by dilution with distilled water. 200 mL of $CuCl_2$ (50mM) solution was mixed with 20 mL of 1% aqueous plant extract and stirred continuously using magnetic stirrer at room temperature for half an hour. The light blue color solution changes to light yellowish green color, which shows the initial formation of copper oxide nanoparticles. Then temperature of solution was raised to 80 ± 2^oC and maintained for 20 min. Now, nearly 20mL of 1M NaOH is added drop by drop and then contents stirred continuously for 30 min. The pH of the solution is 12. After the addition of NaOH causes immediate formation of brownish black precipitate from yellowish green color which indicates the generation of CuO NPs. The precipitate is washed with deionized water followed by ethanol repeatedly for the removal of surface impurities on the particles. The precipitate is dried at 110 ± 2^oC in hot air oven for 2 h. Then the same experiment is repeated to synthesize CuO NPs by using 2%, 3%, 4% and 5% of plant extract respectively. But the consumption of NaOH increases with increase in plant concentration (21, 29, 36, 40 mL for 2%, 3%, 4% and 5% of plant extract respectively). There is no precipitate is observed immediately for 3%, 4% and 5% of plant extract concentration. Fig. 1 shows the graphical abstract of *J. adhatoda* mediated synthesis of CuO NPs.

2.3. Characterization techniques

The X-ray diffraction patterns determine the crystal phases of the synthesized CuO NPs using Bruker Analytical D8 X-ray System with a radiation (Cu- Kα) of 1.54178Å covering the angle between the ranges of $10\text{-}80^0$. The phytogenic synthesized CuO NPs were characterized through Shimadzu UV-Vis Diffuse reflectance Spectrum (DRS) spectrophotometer UV 2600 model and measured in the range between 200-800 nm. Infrared spectrum obtained due to Fourier Transform effect from the spectroscope Shimadzu model: IR affinity-1in the range $400 – 4000cm^{-1}$ using ATR mode was employed to estimate the functional groups present in the biosynthesized of CuO nanoparticles. To understand the morphology and size of the synthesized CuO nanoparticles, High Resolution Transmission Electron Microscopy (HR-

TEM) and Scanning Electron Microscopy (SEM)were studiedusing JEOL Company,JAPAN; Model no.: JEM-2100F and TESCAN VEGA3/Czech Republic respectively.

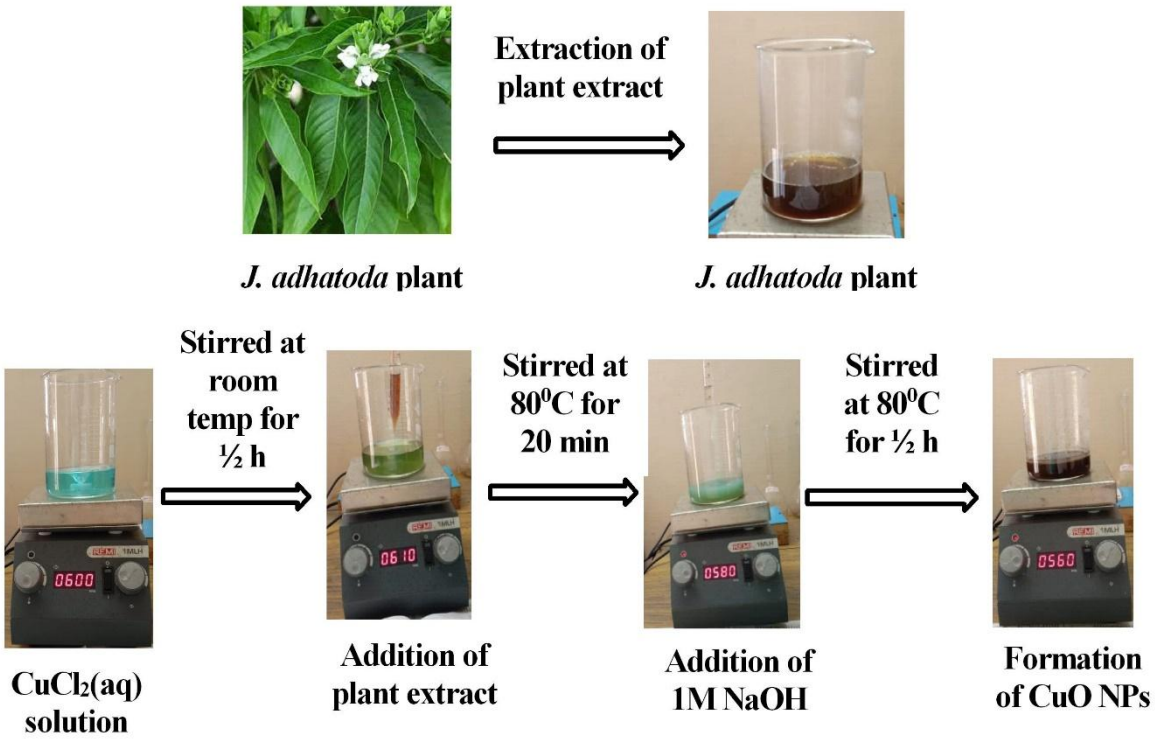

Fig. 1. Flow chart for the synthesis of CuO NPs from aqueous extract of *J. adhatoda* leaves

2.4. Antibacterial activity assay

The obtained CuO NPs was analyzed for antibacterial activity using Agar disc diffusion method. The Bacterial strains of gram-positive bacteria of *Staphylococcus aureus* (strain MTCC 6908), *Bacillus subtilis* (strain MTCC 0441) and gram-negativebacteria of *Escherichia coli* (strain MTCC 1557), *Klebsiella aerogenes* (strain MTCC 0039) were collected from Chandigarh Microbial Type Culture Collection Centre (MTCC), India. The prepared discs with 6mm diameter from Whatman No. 1 filter paper were disinfected at 120±2 °C using autoclave. On completing the disinfection, the discs with moisture were dried on exposing it with hot air oven at 50±2 °C and various concentration (50, 100, 150, 200 & 250 µg/L) of CuO NPs control discs and discs were prepared. The CuO was dissolved in dimethyl sulphoxide (DMSO) and it was used as negative control. Nitrofurantoin (300µg/L) was utilized as positive control. The dried plates were protected at 37±2ºC for 24-36 h.

Succeeding the protection period, the formed zone diameter around the disc paper was taken in millimeter and values are noted. Each experiment was carried out with three triplicates and their average values were documented.

2.5. Antifungal activity assay

The synthesized CuO NPs was treated for antifungal activity using Agar disc diffusion method. The funguses of *Aspergillus fumigatus* (strain MTCC 0343);*Aspergillus niger* (strain MTCC 1344),*Candida albicans (*strain *MTCC 0227)* and *Penicillium chrysogenum* (strain MTCC 0947) were obtained from Microbial Type culture Collection Centre (MTCC), Chandigarh, India. The Potato Dextrose Agar (PDA) loaded with petri dish was sterilized for 20 min and cooled to room temperature. Using sterile swab, the plates with media were seeded with the respective microbial suspension. The discs of 6mm diameter were prepared from filter paper of Whatmann No. 1. The same were disinfected by autoclave at 120$\pm$2°C. After the disinfection, the discs were introduced onto hot air oven at 50$\pm$2 °C for the removal of moisture. Then several concentrations (50, 100, 150, 200 & 250 µg/L) of CuO NPs discs separately were located on each control discs and petri plates were prepared. The CuO NPs was dissolved in DMSO and it was used as negative control. Amphotericin-B (10 µg/L) was used as positive control (standard). The plates were kept at 28$\pm$2 ºC for incubation about 72 hrs. After this process, protection zone is formed and the zone diameter was measured in mm. Each experiment was carried out with three triplicates and their mean values were documented.

3. Results and discussions

3.1 Powder X-Ray diffraction

The samples obtained from synthesis are subjected to XRD and the patterns recorded are shown in the Fig.2. The XRD patterns show the single phasic CuO NPs up to 2% of plant extract concentration. Matching of observed major peaks was found from the X-ray Diffraction pattern of Joint Committee Powder of Standard, JCPDS card number 00-048-1548 is conforming the monoclinic phase of copper oxide nanoparticles. $Na_6Cu_2O_6$ phase was formed as a secondary phase for $\geq$ 3% of plant extract concentration. The major peaks are observed around at 2θ values of 17.1 and 24.2 degree (Fig. 2c) are related to $Na_6Cu_2O_6$. The intensity of peaks increases with increase in plant extract and NaOH concentration. This could be due to excess addition of NaOH with increase in plant extract concentration. A detailed study is needed for further on this issue. From the observation of XRD result, plant concentration of 2% is the optimal concentration for the synthesis of CuO NPsusing *J.adhatoda*. The crystallite size can be arrived using *Scherrer's* formula; D = *Kλ*/

$\beta cos\theta$where, the crystallite size is taken as 'D', the grain sharp factor is assigned to be 'K' (0.94), the incident X-ray wavelength (1.5418 Å) is said to be 'λ' and full width half maximum (FWHM) is assigned to be 'β'. The mean value of crystallite size of the synthesized CuO NPs is calculated and the CuO NPs crystallite size is found to be 40nm.

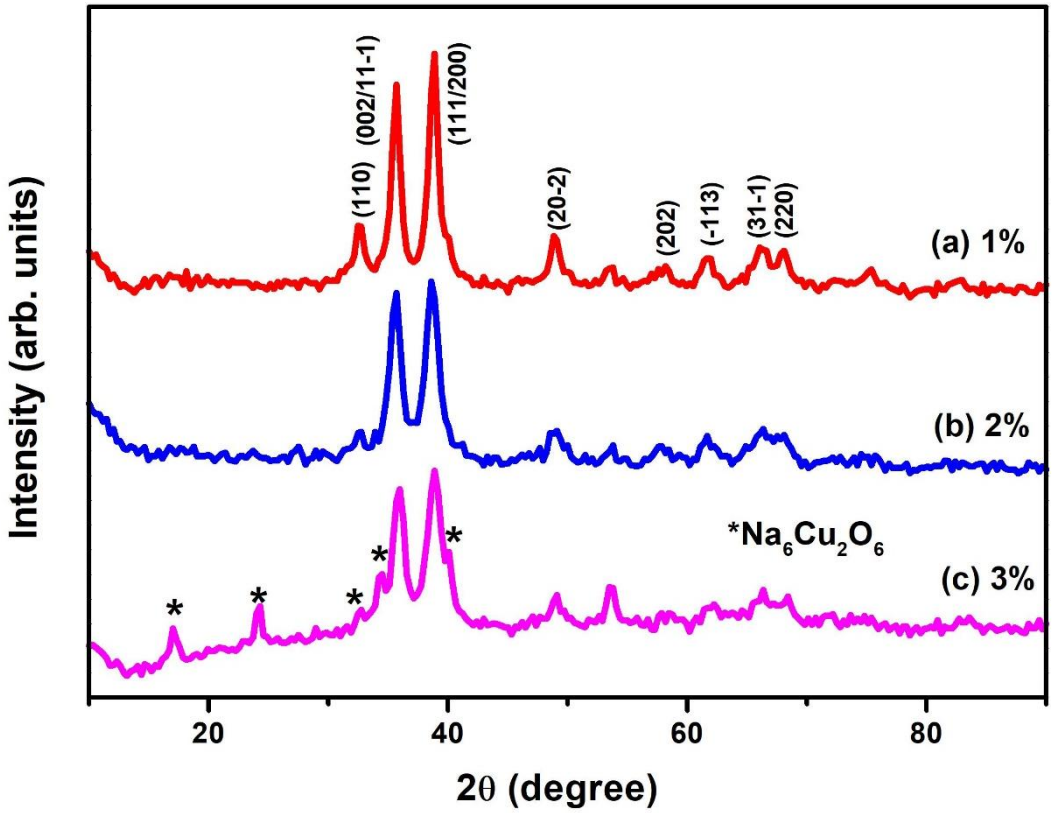

Fig. 2. XRD patterns of CuO NPs synthesized from aqueous extract of J. adhatoda leaves (a) 1%, (b) 2% and (c) 3% concentrations respectively

3.2. UV-Visible spectra

The UV-DRS absorption spectra of the *J.adhatoda* mediated CuO NPs is presented in Fig. 3. A wide absorption peak is obtained around 370 nm shows the formation of CuO NPs. The energy band gap of *J.adhatoda* mediated CuO NPs were calculated using Tauc plot method which was obtained from Kubelka–Munk relation [35]. The band gap was observed as 1.47eV which is closure to the band gap of CuO bulk found in the literature [36]. From the value of band gap, it is suggested that *J.adhatoda* mediated CuO NPs is found to be used as a good visible light photo catalyst.

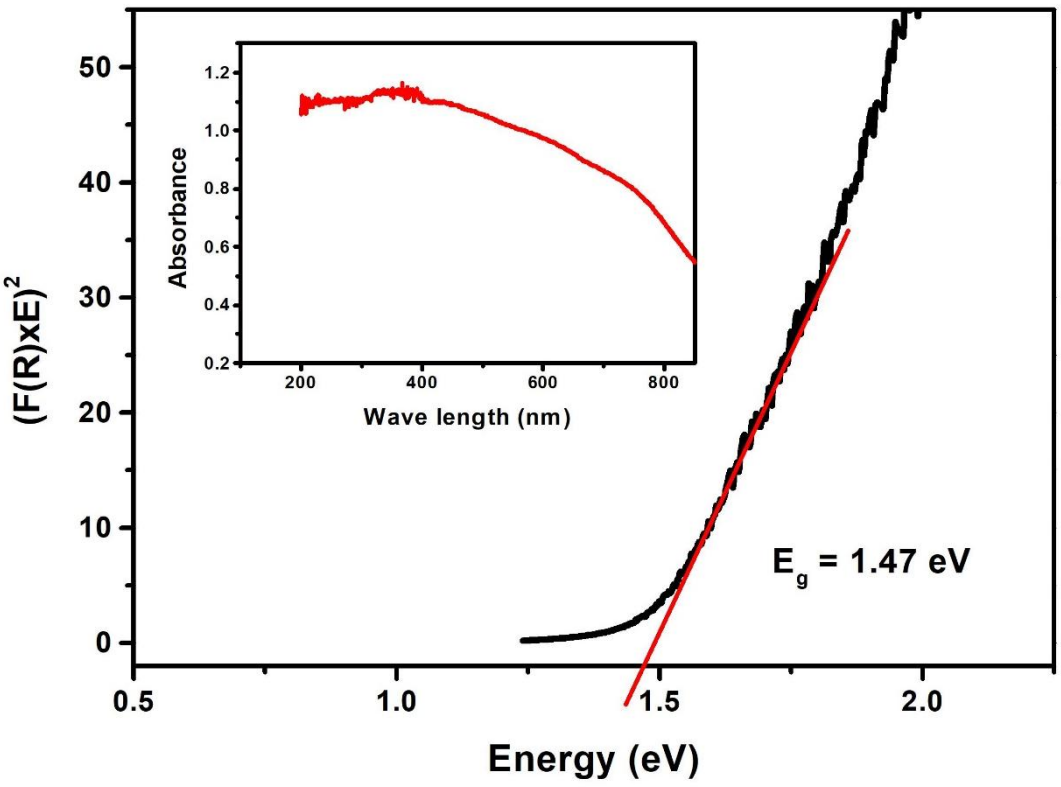

Fig. 3. Tauc plot of CuO NPs with inset of UV-DRS spectra of synthesized CuO NPs from 1% aqueous extract of *J. adhatoda* leaves

3.3. FT-IR spectra

FT-IR spectra of *J. adhatoda* dried leaves powder and CuO NPs synthesized from 1% of *J.adhatoda* leaves extract are depicted in the Fig.4 (a) & (b) respectively. FT-IR spectrum of *J. adhatoda* leaves powder was matched with the result of Ponvel *et al* [37] and Suvidya *et al* [38]. In the Fig.4 (a), a wide band at 3273 cm^{-1} is attributed to OH^- group and sharp peaks at 2920 & 2850 cm^{-1} are assigned to aliphatic -CH_2 and -CH stretching vibrations. A band at 1726 cm^{-1} is related to -C=O group. A broad band at 1608 and 1097 cm^{-1} are ascribed to -NH and -C-O-C- stretching vibrations respectively. In the Fig. 4 (b), a sharp peak at 597, 511 and 430 cm^{-1} respectively are corresponded to typical metal-oxygen vibrations of Cu-O [39, 40]. The peaks at 3441 and 3344 cm^{-1} are related to OH^- group. From result of FT-IR spectra of *J.adhatoda* leaves powder and CuO NPs indicates that the CuO NPs is synthesized from the phyto constituents of *J.adhatoda* leaves extract.

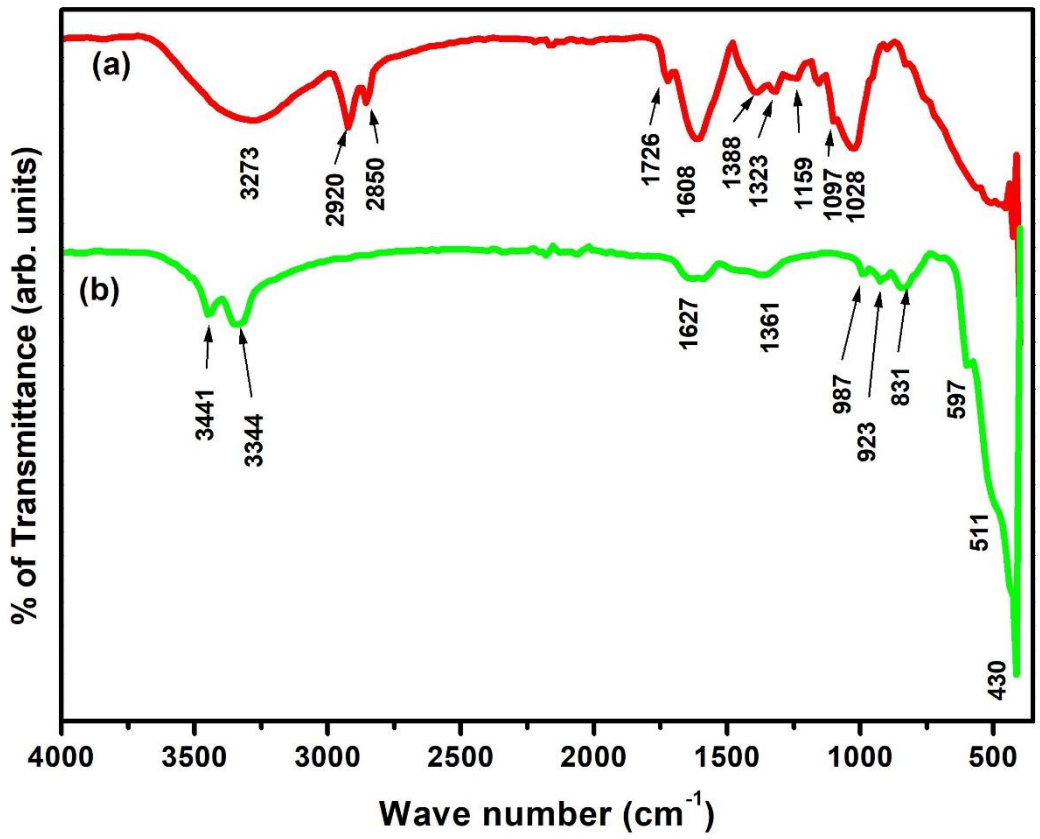

Fig. 4. FTIR spectra of (a) Dried powder of *J. adhatoda* leaves and (b) CuO NPs synthesized from 1% aqueous extract of *J. adhatoda* leaves

3.4. Scanning Electron Microscopy

SEM was utilized to analyze the morphology of nanoparticle. The SEM image of CuO NPs synthesized from 1% plant extract is shown in Fig.5. The particles are in the sphere-shaped morphology and the mean particles size is differing from 40 to 70 nm. Particle size analyzer was performed for CuO NPs synthesized from 2% plant extract (Figure is not shown) and it was observed that the mean value of particle size was around 320 nm. From the observation of SEM and particle size analyzer result, lower concentration of plant extract produces lesser size of nanoparticles.

3.5. High Resolution Transmission Electron Microscopy

A TEM image of CuO NPs synthesized from 1% plant extract is presented in the Fig.6. It is observed that all the particles are uniform size with spherical morphology and free from agglomeration. The mean particles size is differing from 20 to 40 nm.

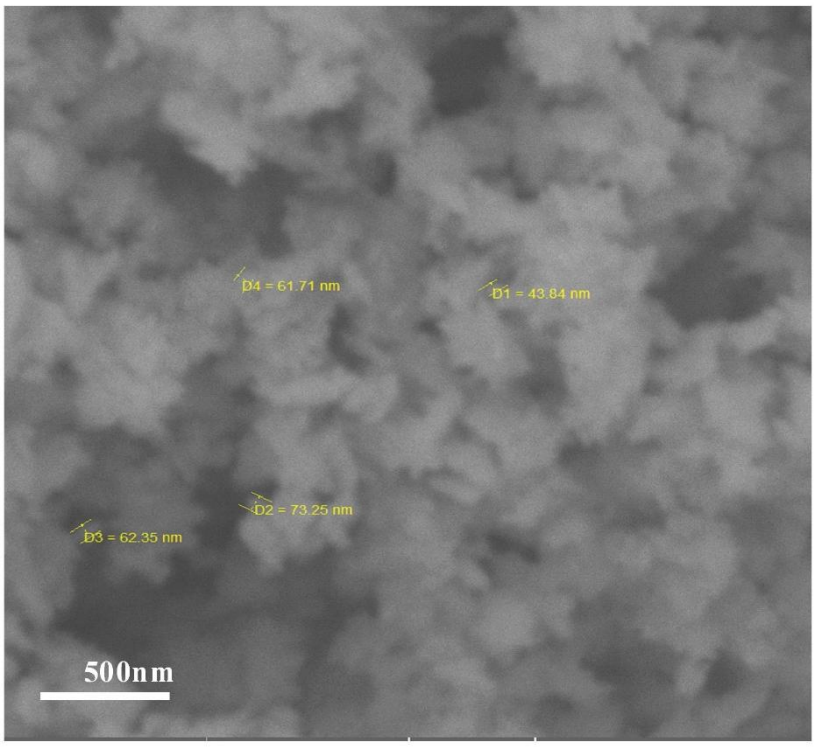

Fig. 5. SEM image ofCuO NPs synthesized from 1% aqueous extract of *J. adhatoda* leaves

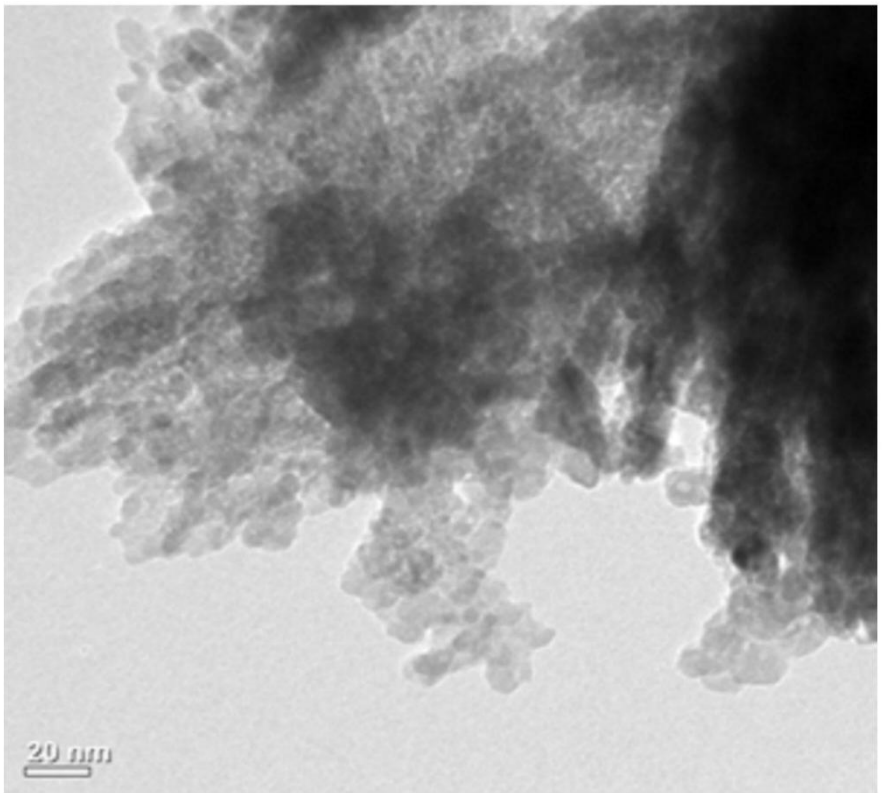

Fig. 6. HR-TEM image ofCuO NPs synthesized from 1% aqueous extract of *J. adhatoda* leaves

3.6. Antibacterial activity of CuO NPs

The antibacterial activity of CuO NPsderived from *J. adhatoda* plant extract was treated against gram-positive bacteria of *Bacillus subtilisStaphylococcus aureus*, and gram-negative bacteria of *Escherichia coli* and *Klebsiella aerogenes*. The zone of inhibition images of bacterial strains is depicted in the Fig.7. The results of CuO NPs antibacterial activity are presented in the Table 1. CuO NPs synthesized from *J. adhatoda* plant extract shows that significant result on antibacterial activity against both Gram- negative and Gram- positive bacteria.The inhibition activity of CuO NPs is represented in bar graph (Fig.8) and maximum zone value (27.2 $\pm$0.9 mm) at 250 µg/Lagainst *E- coli* is observed. This result is significantly higher value for *E- coli* so far reported [41, 42-43]. The antibacterial results show that the gram-positive bacteria are less prone to CuO NPs when compared with the gram-negative bacteria. This activity difference is due to the factors such as structure, composition, variances of cell membrane of the bacteria and morphology of nanoparticles, temperature of synthesis and the duration of interaction with microorganisms of CuO nanoparticles [44-47]. Gram-positive bacteria have heavier murein cell membranes related to the Gram-negative bacteria [48]. The thickening of cell wall of the structure of Gram-positive bacteria leads difficult to penetrate CuO NPs into the cell wall, causing in a low antibacterial activity. The detailed mechanism against antibacterial activity was discussed elsewhere [25]. There are three different mechanisms were discussed based on coordination effects, oxidative stress and non-homeostasis effects that clarify how Cu NPs induce adverse effects on eukaryotic cells. Hou *et al* elaborated the toxicity effect of CuO NPs against microorganism, algae, plants, invertebrates, and vertebrates [49]. From their studies, it is predicted that the small size of nanoparticles can easily penetrate the cell wall through the stomas present in cell membrane or the presence of transporter proteins on the plasma membrane allows the metal ion through it. The entered nanoparticles onto cells via endocytosis interact with oxidative organelles such as mitochondria. Further, redox active proteins activate reactive oxygen species (ROS: OH radicals, O_2^- and H_2O_2) formation in cells and Cu^{2+} ions from the CuO NPs can stimulate ROS by several chemical reactions. ROS can stimulate DNA strand breaks and disturb gene expression. Besides, Cu^{2+} ions have the capacity to form chelates with protein biomolecules forming metalloproteins bonds resulting the inactivation of biomolecules. The Cu^{2+} ions released from CuO NPs increase their local concentration and disrupt cellular metal cation homeostasis to result in cell toxicity. Moreover, the increase in local concentration of Cu^{2+}

ions around the bacterial cell from CuO NPs causes disruption of cellular metal cation homeostasis to outcome of cell toxicity.

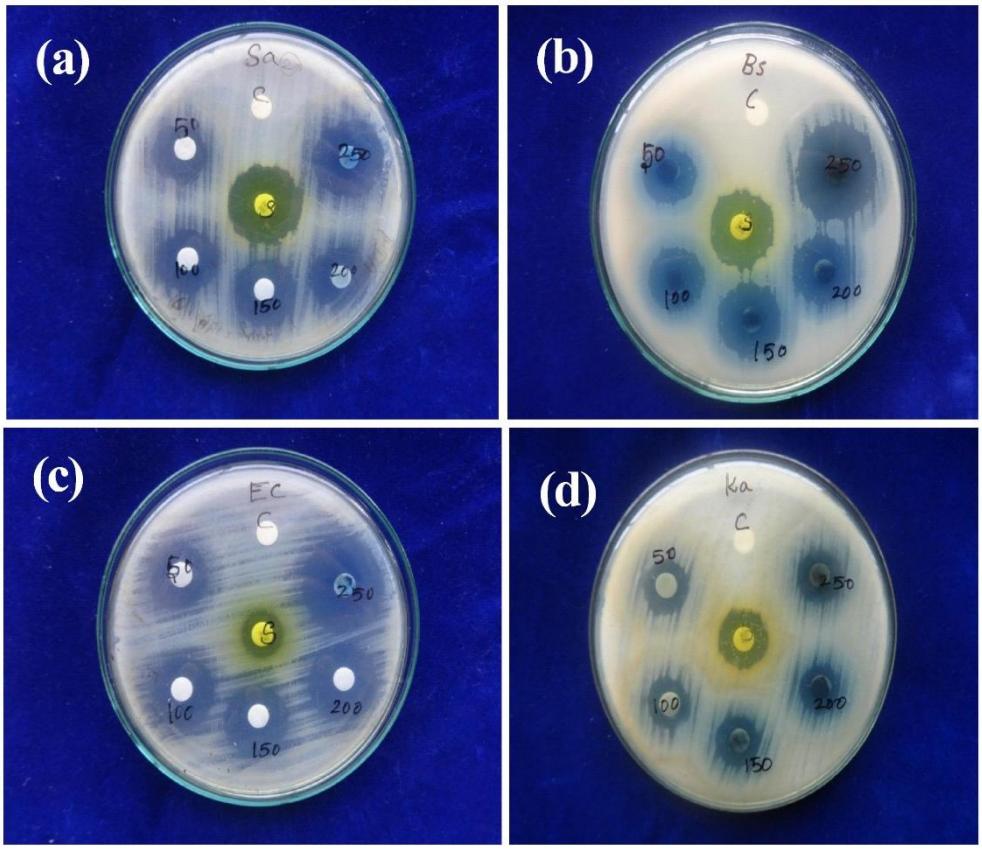

Fig. 7. Zone of inhibition of CuO NPs against bacteria (a) *Staphylococcus aureus*, (b) *Bacillus subtilis*, (c) *Escherichia coli* and (d) *Klebsiella aerogenes*

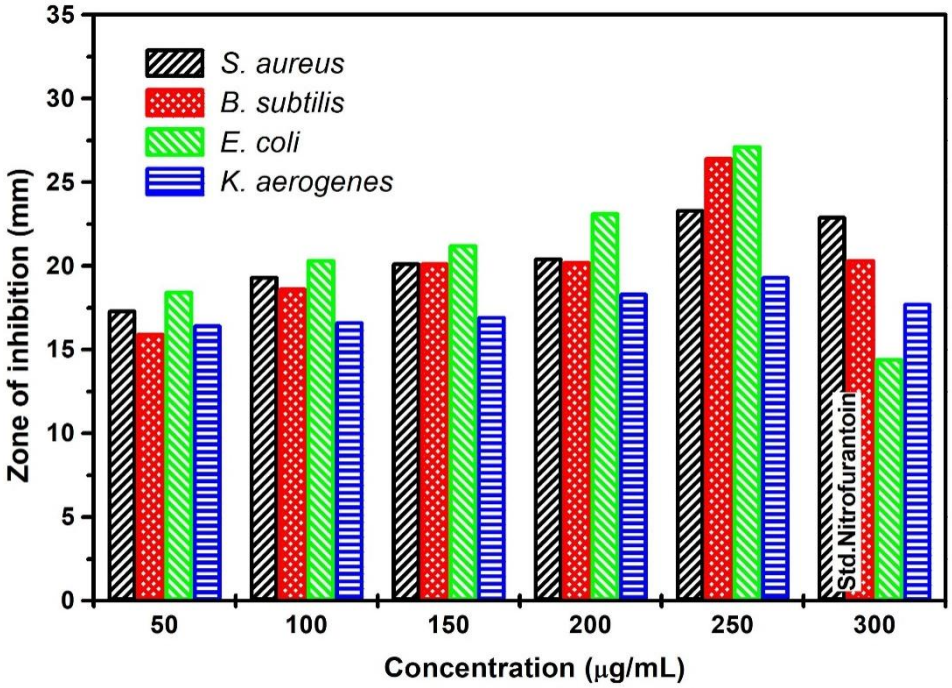

Fig. 8. Bar graph representing Zone of inhibition of CuO NPs against various bacteria

Table 1. Antibacterial activity of CuO NPs with variable concentrations

Concentration	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella aerogenes*
	Zone of Inhibition (diameter in mm)			
50µg/L	17.4 ±1.0	16.0 ±1.7	18.5 ±1.1	16.5 ±0.8
100 µg/L	19.4 ±1.1	18.7 ±0.7	20.4 ±1.1	16.7 ±0.8
150 µg/L	20.2 ±0.6	20.2 ±1.4	21.3 ±0.9	17.0 ±0.5
200 µg/L	20.5 ±1.0	20.3 ±1.1	23.2 ±0.6	18.4 ±0.9
250 µg/L	23.4 ±1.1	26.5 ±0.8	27.2 ±0.9	19.4 ±1.0
Std -300 µg/L	23.0 ±0.7	20.4 ±1.0	14.5 ±0.7	17.8 ±0.7

3.7. Antifungal activity of CuO NPs

The antifungal activity of *J. adhatoda* plant extract derived CuO NPs was tested against the funguses of *Aspergillus fumigatus*,*Aspergillus niger*,*Candida albicans and Penicillium chrysogenum*. The zone of inhibition images of fungal strains is shown in the Fig. 9. The effects of the antifungal activity of CuO NPs are presented in the Table 2. A bar graph is representing the inhibitory activity against various fungal stains is shown in the Fig. 10. Maximum zone of inhibition (17.7 ±0.6 mm) is observed for *P. chrysogenum* at 250 µg/L which is higher than that of positive control Amphotericin-B. The results obtained for antifungal activities are comparable with previous study reported by Vanathi *et al* [50].The mechanisms of inhibition growth of fungi by CuO NPs are very limited. Safaeia *et al* presented the detailed mechanism for funguses [51]. They inferred that nanoparticles can enter the cell wall of the fungi via pores on the surface and can destroy the cell membrane by resulting inactivity of fungi. Moreover, metal oxide NPs can induce oxidative stress on cell membrane due to the production of ROS and disturbing the stability of cell which finally causing fungal death. Cu^{2+} ions produced from the CuO NPs may diffuse through cell wall and generate stress that consequently leads to death of fungi by interrupting the respiratory dehydrogenation and electron transfer chain. The role of all intercellular organelles such as lysosome, endoplasmic reticulum, ribosome, and mitochondria were interrupted due to the diffusion of CuO NPs into the fungi by producing ROS. The effective antifungal activity was observed for the nanoparticles are having the size lesser than 50 nm. These nanoparticles are

easily transferring metal ions through the pores of the nucleic membrane and attaching to DNA, which interrupt the processes such as transcription, replication and translation and finally undergo fungal death. Further investigations are required to understand much deeper expertise the mechanism of antifungal activity of CuO NPs.

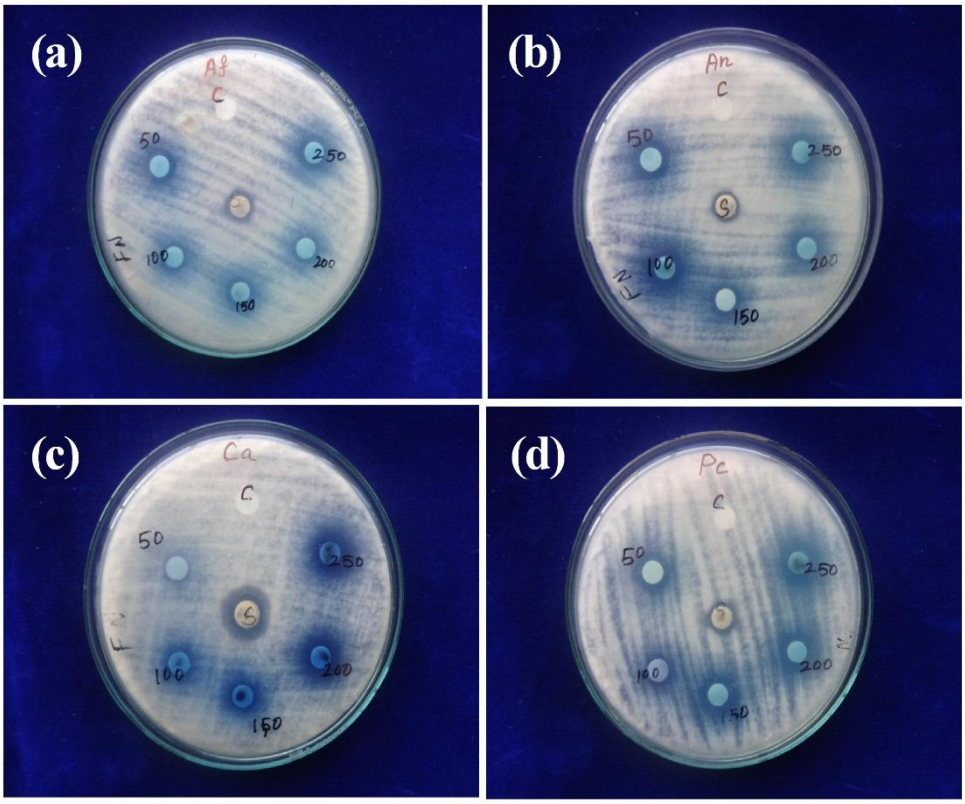

Fig. 9. Zone of inhibition of CuO NPs against fungus (a) *Aspergillus fumigatus*, (b) *Aspergillus niger*, (c) *Candida albicans* and (d) *Penicillium chrysogenum*

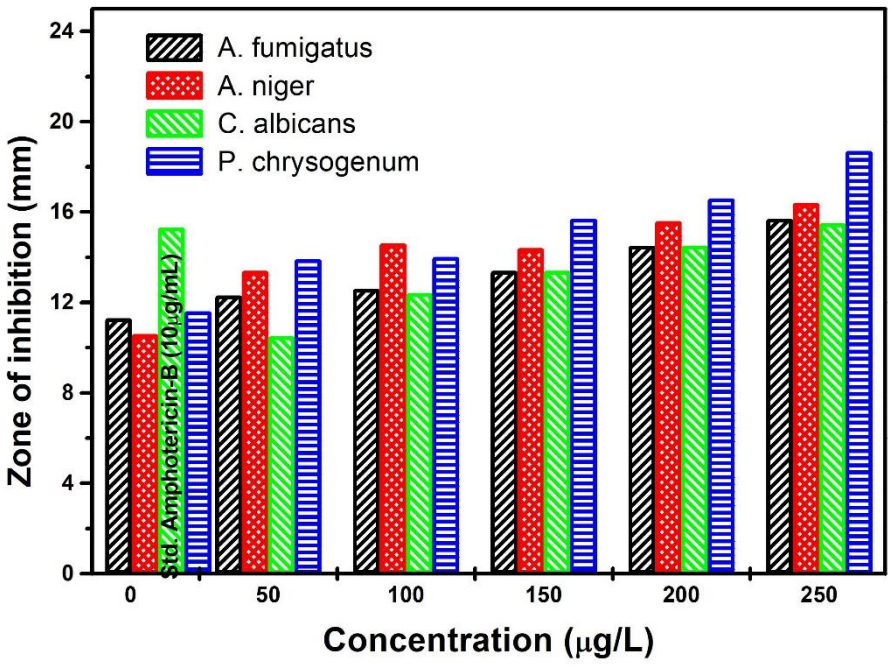

Fig. 10. Bar graph representing Zone of inhibition ofCuO NPs against various funguses

Table 2. Antifungal activity of CuO NPs with variable concentrations

Concentration	*Aspergillus fumigatus*	*Aspergillus niger*	*Candida albicans*	*Penicillium chrysogenum*
	Zone of Inhibition (diameter in mm)			
50 µg/L	12.3 ±1.2	13.4 ±0.8	10.5 ±0.7	13.7 ±1.4
100 µg/L	12.6 ±1.2	14.6 ±1.1	12.4 ±1.0	14.0 ±1.7
150 µg/L	13.4 ±1.1	14.4 ±1.2	13.4 ±0.8	15.7 ±0.6
200 µg/L	14.5 ±1.1	15.6 ±1.0	14.5 ±1.0	16.6 ±0.9
250 µg/L	15.7 ±1.0	16.4 ±1.0	15.5 ±1.0	17.7 ±0.6
Std -10 µg/L	11.3 ±1.1	10.6 ±0.8	15.3 ±1.0	11.6 ±1.5

4. Conclusions

The current study reported the synthesis of CuO NPs using aqueous extract of *J. adhatoda* leaves with various concentrations. Powder XRD patterns confirmed the single phasic CuO NPs up to 2% concentration of plant extract. Above 2% plant extract concentration produced the secondary phase of $Na_6Cu_2O_6$. UV-DRS spectra agreed the result of Powder XRD and the band gap was 1.47 *eV*. The peaks were observed in FT-IR spectra of both plant leaves and CuO NPs substantiated that the CuO NPs were synthesized from the bio reduction by the aqueous extract of *J. adhatoda* leaves. SEM image showed that the particles were in spherical shaped with uniform morphology. TEM image reported that the spherical morphology of CuO NPs with the particles sizes was differing from 20 to 40 nm. Antimicrobial activity of CuO NPs was good agreement against bacteria and funguses. Maximum zone of inhibition was 27.2 ±0.9 mm against *E. coli* and 17.7 ±0.6 mm against *P. chrysogenum* at 250 µg/L respectively for antibacterial and antifungal activity of CuO NPs. This study was successfully synthesized the CuO NPs through cost effective and environment friendly approach and moreover CuO NPs could be used a potential candidate for good antibacterial and antifungal applications.

References

[1] Md. Nasrollahzadeh, M. Maham, S.Md. Sajadi, "Green synthesis of CuO nanoparticles by aqueous extract of *gundelia tournefortii* and evaluation of their catalytic activity for the synthesis of n-monosubstituted ureas and reduction of 4- nitrophenol", *J. Colloid and Interface Science,* 455, 245 (2015).

[2] J. K. Sharma, M. Shaheer Akhtar, S. Ameen, "P. Srivastava and G. Singh, "Green synthesis of CuO nanoparticles with leaf extract of *calotropis gigantea* and its dye-sensitized solar cells applications", *J. Alloys and Compds.,* 632, 321 (2015).

[3] R. Sankar, P. Manikandan, V. Malarvizhi, T. Fathima, K. S. Shivashangari, V. Ravikumar, "Green synthesis of colloidal copper oxide nanoparticles using *carica papaya* and its application in photocatalytic dye degradation", Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 121,746 (2014).

[4] K. R. Reddy,"Green synthesis, morphological and optical studies of CuO nanoparticles", *J. Molecular Structure,* 1150, 553(2017).

[5] D. D. Gültekin, A. A. Güngör, H. Onem, A. Babagil, H. Nadaroğlu, "Synthesis of Copper Nanoparticles Using a Different Method: Determination of Their Antioxidant and Antimicrobial Activity", *J. Turkish Chemical Society A.,* 3(3), 623 (2016).

[6] V. Gnanavel, V. Palanichamy, S. Mohana Roopan, "Biosynthesis and characterization of copper oxide nanoparticles and its anticancer activity on human colon cancer cell lines (HCT-116)", *J. Photochemistry & Photobiology, B: Biology,* 17, 133(2017).

[7] Y. Abboud , T. Saffaj ,A. Chagraoui ,A. El Bouari , K. Brouzi , O. Tanane ,B. Ihssane, "Biosynthesis, characterization and antimicrobial activity of copper oxide nanoparticles (CuO NPs) produced using brown alga extract (*Bifurcaria bifurcate",Appl. Nanosci*., 4, 571(2014).

[8] M. Aziz, S. Saber Abbas, W. Rosemaria, W. Baharom, "Size-controlled synthesis of SnO_2 nanoparticles by sol–gel method", *Materials Letters,* 91, 31(2013).

[9] R. Vijayakumar, R. Elgamiel, Y. Diamant, A. Gedanken, "Sonochemical preparation and characterization of nanocrystalline copper oxide embedded in poly (vinyl alcohol) and its effect on crystal growth of copper oxide", *Langmuir,* 17, 1406 (2001).

[10] C.L. Carnes, J. Stipp, K.J. Klabunde, "Synthesis, characterization, and adsorption studies of nanocrystalline copper oxide and nickel oxide", *Langmuir*, 18, 1352 (2002).

[11] A.J. Yin, J. Li, W. Jian, J. Bennett, J.H. Xu, "Fabrication of highly ordered metallic nanowire arrays by electrodeposition", *Appl. Phys. Lett.,* 79(7), 1039 (2001).

[12] H. Fan, L. Yang, W. Hua, X. Wu, Z. Wu, S. Xie, B. Zou, "Controlled synthesis of monodispersed cuo nanocrystals", *Nano-technology*, 15, 37 (2004).

[13] P. Sutradhar, M. Saha, D. Maiti, "Microwave synthesis of copper oxide nanoparticles using tea leaf and coffee powder extracts and its antibacterial activity", J. *Nanostruct. Chem*,4, 86 (2014).

[14] J.F. Xu, W. Ji, Z.X. Shen, S.H. Tang, X.R. Ye, D.Z. Jia X.Q. Xin, "Preparation and characterization of CuO nanocrystals", *J. Solid State Chem*., 147, 516 (1999).

[15] M. Salavati,F. Davar, "Synthesis of Copper and Copper (I) oxide nanoparticles by thermal decomposition of a new precursor", *Mater. Lett*., 63, 441(2009).

[16] G. Marslin, K. Siram, Q. Maqbool, R. Kamalabai Selvakesavan, D. Kruszka, P. Kachlicki and G. Franklin, "Secondary metabolites in the green synthesis of metallic nanoparticles", *Materials*, 11, 940 (2018).

[17] A.M. Awwad, B.A. Albiss, N.M. Salem, **"**Antibacterial activity of synthesized copper oxide nanoparticles using *malva sylvestris* leaf extract", *SMU Medical J*., 2(1), 91 (2015).

[18] L. B. Shi, P. F. Tang, W. Zhang, Y. P. Zhao, L. C. Zhang, and H. Zhang, "Green synthesis of CuO nanoparticles using *Cassia auriculata* leaf extract and in vitro evaluation of their biocompatibility with rheumatoid arthritis macrophages", *Tropical J. Pharmaceutical Research*, 16 (1), 185 (2017).

[19] B. Turakhia, M. B. Divakara, M. S. Santosh, S. Shah, "Green synthesis of copper oxide nanoparticles: a promising approach in the development of antibacterial textiles", *J. Coat. Technol. Res*. (2020).

[20] D.D. Priya, S.M. Roopan, S. Singh, J. Bansal, S. Shanavas, M.R. Khan, N.A. Al-Dhabi, M.V. Arasu, V. Duraipandiyan, "Phyto-synthesis of CuO nano-particles and its catalytic application in C-S bond formation", *Materials Letters* (2020),doi.org/10.1016/j.matlet.2020.127486.

[21] G. Borkow, J. Gabbay, "Copper: an ancient remedy returning to fight microbial, fungal and viral infections", *Curr.Chem.Biol*, 3, 272 (2009).

[22] P. Ponmurugan, K. Manjukarunambika,V. Elango, B. Mythili Gnanamangai, "Antifungal activity of biosynthesized copper nanoparticles evaluated against red root-rot disease in tea plants", *J. Experimental Nanoscience*, 11:13, 1019 (2016).

[23] P.C. Garcıa, R. M. Rivero, J. M. Ruiz, "The role of fungicides in the physiology of higher plants: implications for defense responses". *Bot Rev*, 69, 162 (2003).

[24] G. Sçnchez-Sanhueza., D, Fuentes-Rodrêguez, H. Bello-Toledo,"Copper nanoparticles as potential antimicrobial agent in disinfecting root canals. A systematic Review", *Int. J. Odontostomat*., 10(3), 547 (2016).

[25] A. P. Ingle, N. Duran, M. Rai, "Bioactivity, mechanism of action, and cytotoxicity of copper-based nanoparticles: A review", *Appl. Microbiol. Biotechnol*., 98, 1001 (2014).

[26] G. Isani, M. Falcioni, G. Barucca, "Comparative toxicity of CuO nanoparticles and $CuSO_4$ in rainbow trout", *Ecotoxicol. Environ. Saf*.,97, 40 (2013).

[27] G. Sçnchez-Sanhueza, D. Fuentes-Rodrêguez, H. Bello-Toledo, **"**Copper nanoparticles as potential antimicrobial agent in disinfecting root canals. A systematic Review". *Int. J. Odontostomat.*, 10(3), 547 (2016).

[28] A.F. O. Azo, T. Nakama, M. Nakamura, T. Futagami, M. Catherine, M. Vestergaard, "Antifungal Potential of Nanostructured Crystalline Copper and Its Oxide Forms", *Nanomaterials*, 10, 1003 (2020).

[29] Rashmi Pa, L. Mathew, "Antimicrobial activity of leaf extracts of *Justicia adhatoda* L. in comparison with vasicine*", Asian Pacific J. Tropical Biomedicine*, S1556 (2012).

[30] R.Malathi, D.Kaviyarasan, S.Chandrasekar, "Preliminary Phytochemical Analysis Of *JusticiaAdhatoda* leaves Extract Using Different Solvents", *Int. J. Pharm. Drug. Anal*, 6(2), 186 (2018).

[31] M. Selvarani, V. Jeyasimga, "Phytochemical Screening and Bactericidal Potency of *Justicia Adhatoda* Against Clinical Pathogens", *Asian J Pharm Clin Res*, 13(10), 72 (2020).

[32] P.G. Bhavyasree, T.S. Xavier. "Green synthesis of Copper Oxide/Carbon nanocomposites using the leaf extract of Adhatoda vasica Nees, their characterization and antimicrobial activity". *Heliyon* 6 (2020) e03323.

[33] K. S. Dayana, R. J. Mani, S. C. Vella Durai, "Green synthesis of CuO nanoparticle using *Justicia Adhatoda* leaf extract and it's antibacterial and thermal asset". *Digest J. of Nanomaterials and Biostructures*, 16 (3), (2021), 1119 – 1126.

[34] Md. J. Islam, M. T. Khatun, Md. R. Rahman, and M. M. Alam. "Green synthesis of copper oxide nanoparticles using Justicia adhatoda leaf extract and its application in cotton fibers as antibacterial coatings". *AIP Advances* 11, 125223 (2021)

[35] S. Landi Jr, I. R. Segundo, E. Freitas, M. Vasilevskiy, J. Carneiro, C.J. Tavares. "Use and misuse of the Kubelka-Munk function to obtain the band gap energy from diffuse reflectance measurements". *Solid State Communications* 341 (2022) 114573.

[36] N. R. Dhineshbabu1, V. Rajendran, N. Nithyavathy, R. Vetumperumal. Study of structural and optical properties of cupric oxide nanoparticles. *Appl Nanosci.,* (2016) 6:933–939.

[37] K. M. Ponvel, T. Narayanaraja, J. Prabakaran, "Biosynthesis of Silver nanoparticles using root extract of the medicinal plant *Justicia adhatoda*: Characterization, electrochemical behavior and applications", Int. *J. Nano Dimens.,* 6(4), 339 (2015).

[38] R. Suvidya, L. Pravin, B. Shubhangi, J. Satyawati, "Rapid Green Synthesis and Biological Activity of Silver Nanoparticles Using *Adhatoda vasica* Leaf Extract", *Advanced Science,Engineering and Medicine,* 5, 1(2012).

[39] A. V. Singh, R. Patil, A. Anand, P. Milani and W.N. Gade**, "**Biological Synthesis of Copper Oxide Nano Particles Using *Escherichia coli", Current Nanoscience,* 6, 365 (2010).

[40] M. S. Jadhav, S.Kulkarni, , P. Raikar, D. A. Barretto, S. K. Vootla, U. S. Raikar, "Green biosynthesis of CuO & Ag–CuO nanoparticles from Malus domestica leaf extract and evaluation of antibacterial, antioxidant and DNA cleavage activities", *New Journal of Chemistry*,42(1), 204 (2018).

[41] K. Lingaraju, H. R. Naika, K. Manjunath, G. Nagaraju, D. Suresh, H. Nagabhushana, "*Rauvolfia serpentina*-mediated green synthesis of CuO nanoparticles and its multidisciplinary studies", *Acta Metallurgica Sinica*, 28(9), 1134 (2015).

[42] F. Ijaz1, S. Shahid, S. Ahmad Khan, W. Ahmad, S. Zaman, "Green synthesis of copper oxide nanoparticles using *Abutilon indicum* leaf extract: Antimicrobial, antioxidant and photocatalytic dye degradation activities", *Tropical Journal of Pharmaceutical Research*, 16 (4), 743 (2017).

[43] M. Altikatoglu, A. Attar, F. Erci, C. M. Cristache, I. Isildak, "Green synthesis of copper oxide nanoparticles using *ocimum basilicum* extract and their antibacterial activity", *Fresenius Environmental Bulletin* 26(12A), 7832 (2017).

[44] H. Raja Naika, K. Lingaraju, K. Manjunath, Danith Kumar, G. Nagaraju, D.Suresh, H. Nagabhushana, "Green synthesis of CuO nanoparticles using *Gloriosa superba* L. extract and their antibacterial activity", *Journal of Taibah University for Science*, 9(1), 7 (2014).

[45] M. Heinlaan, A. Ivask, I. Blinova, H. C. Dubourguier, A. Kakru, "Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus", *Chemosphere*,71(7), 1308 (2008).

[46] X. Liang, M. Sun, L. Li, R. Qiao, K. Chen, Q. Xiao, F. Xu, "Preparation and antibacterial activities of polyaniline/Cu0.05Zn0.95O nanocomposites", Dalton Trans., 41(9), 2804 (2012).

[47] A. Azam, A. S. Ahmed, M. Oves, M. S. Khan, A. Memic, "Size-dependent antimicrobial properties of CuO nanoparticles against Gram-positive and -negative bacterial strains", *Int J. Nanomedicine*, 7(9), 3527 (2012).

[48] J. S. Tawale, K. Dey, R. Pasricha, K. N. Sood, A. K. Srivastava, "Synthesis and characterization of ZnO tetrapods for optical and antibacterial applications", *Thin Solid Films*, 519 (3), 1244 (2010).

[49] J. Hou, X. Wang, T. Hayat, X. Wang, Ecotoxicological effects and mechanism of CuO nanoparticles to individual organisms, *Environ. Pollut*. 221 209 (2017).

[50] P Vanathi, P Rajiv, and R. Sivaraj, "Synthesis and characterization of *Eichhornia*-mediated copper oxide nanoparticles and assessing their antifungal activity against plant pathogens", *Bull. Mater. Sci*., 39(5), 1165 (2016).

[51] M. Safaeia, M. Taranb, M. M. Imani, "Preparation, structural characterization, thermal properties and antifungal activity of alginate-CuO bio nanocomposite", *Materials Science & Engg. C.*, 101, 323 (2019).