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Green Synthesis, Characterization and Antimicrobial Activity Studies of Salicylalchitosan Biofunctionalized Copper Oxide Nanoparticles

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In the present work, we reported the green synthesis of biofunctionalized copper oxide nanoparticles with salicylalchitosan derived from chitosan. Salicylalchitosan, copper oxide nanoparticles and biofunctionalized nanoparticles were characterized by UV-visible, FT-IR, SEM and TEM techniques and antimicrobial activity studies of those were undertaken. The size of synthesized copper oxide nanoparticles was in the ranges around 60 to 100 nm and bio functionalized particles were about 100 nm. The antimicrobial activities of biofunctionalized copper oxide nanoparticles were observed higher inhibition activity than the non-functionalized nanoparticles and salicylalchitosan against standard microbial species.

Keywords: Chitosan, Salicylalchitosan, Copper oxide, Nanoparticles, Green synthesis, Antimicrobial activities.

INTRODUCTION

In the last few years bionanotechnology has attracted large number of scientists from biology, physics, chemistry, materials sciences and also from applied sciences such as medicine and biotechnology. Biofunctionalization of nanoparticles can provide good biocompatibility for the immobilization of biomolecules, tissues or cells and high specificity for biological recognition which led to produce a considerable effect in biological systems. Therefore, biofunctionalized nanomaterials are being given considerable attention in a multiple way of emerging fields of science and technology [1-5].

However, seeking suitable biomaterials for the functionalization of nanomaterials is one of the key issues in this hottest field. Chitosan is natural, eco-friendly potential bioactive polymer. The development of new applications for chitosan and its derivative is due to the fact that these are renewable source of natural biodegradable, biocompatible polymers. Currently, chitosan has been attracted more attention for its unique physico-chemical characters, versatility, non-toxic, economical, easy availability and bioactivities [6-8].

Copper nanoparticles have concerned considerable attention because they are very reactive and their high surface-to-volume ratio helps to interact with bacterial surface effectively. Moreover its low-cost, high yields and short reaction times under normal reaction conditions are the advantages in green-nano preparation. The copper nanoparticles were prepared through various green methodologies and proved its superior antimicrobial activity against various bacterial and fungal strains from many researches [9-12].

From environmental point of view, plant extracts used reduction methods can be considered as more effective green approaches for synthesizing metal nanoparticles, because of employing plants towards synthesis of nanoparticles are emerging as advantageous compared to others [13]. In the present investigation, we have utilized lemon extract and curcumin extract in the preparation of copper oxide nanoparticles. Lemons are a rich source of citric acid and ascorbic acid, easily available material and also known for its water softening properties. This extracts act as a good metal reductant in the nanoparticle preparation according to some research works [14,15]. Curcumin are polyphenols, an active component of turmeric plant and curcumin stabilized metal nanoparticles have been shown to have a wide range of therapeutic effects [16-18].

Based upon the above discussion, this study was investigated to find the highly active microbial agents in the different way of synthesis by the biofunctionalization of salicylalchitosan with copper oxide nanoparticles. Firstly, we have prepared the bioactive salicylalchitosan by using chitosan and on other hand copper oxide nanoparticles have been prepared by using lemon extract as a reductant and curcumin as a capping agent. Finally

salicylalchitosan was functionalized with copper oxide nanoparticles separately. Salicylalchitosan, copper oxide nanoparticles and biofunctionalized nanoparticles were analyzed for antimicrobial activity against various Gram-positive and Gram-negative bacterial species and some fungal species and found the inhibition results of biofunctionalized nanomaterials were more appreciable.

EXPERIMENTAL

All the chemicals and solvents used were of analytical reagent grade and obtained from Merck (India) Ltd. The prawn shells material was obtained from local sea food markets at Tuticorin, India. The turmeric sample (BSR-01) was obtained from Agricultural College and Research Institute, Madurai, India.

The UV-visible absorption spectra of the samples were measured on a Shimadzu UV-visible V-530A spectrophotometer in the range of 300 to 900 nm. FT-IR spectra analysis was recorded on a Jasco FT-IR/4100 spectrophotometer in the range of 4000 to 400 cm^{-1}. SEM images were recorded by using JEOL Model JSM-6390LV scanning electron microscope. High resolution transmission electron microscopy (HRTEM) was carried out using a 300 KV JEOL-3011 instrument with an ultrahigh resolution pole piece to determine the morphological changes.

Collection of extracts: Lemon fruits were collected from the local markets and washed, cut into pieces and squeezed well to make 5 to 10 mL pure extract. The extract was then filtered using Whatman's No. 1 filter paper. The filtrate was collected in a clean dried container and it was stored for further uses.

Curcumin was quantitatively extracted from turmeric in Soxhlet apparatus by using 95 % ethanol and the curcumin content was estimated according to Manjunath *et al*. [19]. Turmeric dried powder weighed (5 g) and taken in Soxhlet apparatus and 250 mL of ethanol was poured into the apparatus. The extraction process was carried out for 2-3 h and the final ethanol residual curcumin extract was evaporated and dried.

Isolation of chitosan from prawn shells: According to the preparation method of Brine and Austin [20]; Muzzarelli *et al.* [21], chitosan was isolated from prawn shells of chitin source. Double distilled water has been used throughout the synthesis process. Prawn shells were washed several times and dried. The dried powdered material was demineralized by 1 M hydrochloric acid solution with constant stirring and the solution was washed by double distilled water and dried in oven at 60 °C for 3-4 h. Again this dried powder was deprotienized with 1M aqueous sodium hydroxide with constant stirring and kept in hot magnetic stirrer at 60 °C for 1 h. The pink colour solution was obtained and washed several times with double distilled water and dried in oven at 60 °C overnight. The dried chitin powder was deacetylated with aqueous solution of 40 % sodium hydroxide for 2 h at 60 °C and the obtained dirty whitish precipitate of chitosan was washed several times and dried in oven at 60 °C for 3-4 h.

Synthesis of salicylalchitosan (SC): The synthesis process was followed according to the preparation method of Jayandran and Haneefa [22]. Double distilled water has been used throughout the synthesis process. 100 mg of synthesized dried chitosan powder was dissolved in 25 mL of acetic acid solution (0.5 M) and kept in magnetic stirrer for 2 h at room temperature to get complete dissolved solution. Then another mixture of 10 mL of salicylaldehyde (0.1 M) in ethanol was prepared and stirred well for 1 h at room temperature. The ethanolic mixture of salicylaldehyde was added to chitosan mixture and kept in hot magnetic stirrer at 50-60 °C for 12 h and refluxed well. The obtained yellowish green coloured precipitate of salicylalchitosan was cooled and filtered then washed several times with ethanol and dried under vacuum at 60 °C for 12 h. The synthesized yellowish green powder was kept in a desiccator over silica gel for further analyses.

Synthesis of copper oxide nanoparticles (CONPs): 1 mM aqueous solution of copper chloride was prepared for the synthesis of CONPs. The freshly prepared copper solution (10 mL) was mixed with fresh lemon extract (10 mL) with constant stirring for the reduction of copper metal. The reaction mixture was kept in the magnetic hot stirrer at 50-60 °C for a particular time to colour change from pale bluish green to pale green which denoted the metal reduction. The above mixture was mixed with freshly prepared curcumin extract (1 mM) and the stirring was continued. The solution colour was changed from yellow to yellowish brown slowly and finally a permanent dark brown colour which indicated the complete stabilization of CONPs. The reaction pH was maintained in between 3-4 and temperature was at 50-60 °C throughout the experiment. The solution was centrifuged with washing several times to obtain the pure CONPs. The supernatant layer was decanted and kept in oven.

Biofunctionalization of copper oxide nanoparticles (BCONPs): In this scheme biofunctionalization of salicylalchitosan with copper oxide nanoparticles were carried out by the normal interaction. 1 mM solution of CONPs (10 mL) added with 1mM of salicylalchitosan (10 mL) with constant stirring at 60 °C temperature for 1 h. The reddish brown colour was obtained and the reaction was continued for 1 h. Finally, the brown colour solution was obtained which denoted the strong functionalization of salicylalchitosan with copper oxide nanoparticles.

Biological assay: The antibacterial activity of the samples were tested by disc diffusion method against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), two Gram-negative bacteria (*Escherichia coli* and *Salmonella typhi*) and antifungal activity was carried out by agar well diffusion method against four fungi (*Candida albicans*, *Curvularia lunata*, *Aspergillus niger* and *Trichophyton simii*).

For disc diffusion method [23], stock cultures incubated in nutrient agar were transferred to test tube of Muller-Hinton broth for bacteria that were incubated for 24 h at 37 °C. The cultures were diluted with fresh Muller-Hinton broth to get 2×10^6 CFU/mL for bacteria. The Muller Hinton Agar plates were prepared by pouring 15 mL of molten media into sterile petri plates. The sample was loaded, placed on the surface of the cultured agar plates and incubated at 37 °C for 24 h then inhibition zones formed around the disc were measured. The results were compared with standard antibiotic, chloramphenicol.

For agar well diffusion method [24], the fungal strains were suspended in Sabouraud's dextrose broth for 6 h to give

concentration 10^5 CFU/mL and then inoculated with the culture medium. A total of 8 mm diameter wells were punched into the agar and filled with the sample and solvent blanks (hydro alcohol and hexane). Standard antibiotic, Fluconazole (concentration 1 mg/mL) was used as positive control and fungal plates were incubated at 37 °C for 72 h. The diameters of zone of inhibition observed were measured.

RESULTS AND DISCUSSION

UV-visible studies: The UV-visible spectra of salicylalchitosan are given in Fig. 1a. Salicylalchitosan exhibits the absorption bands in the UV spectrum at 355 nm with low intensities. This absorption band could be assigned to π-π* and n-π* transitions in the aromatic ring or azomethine.

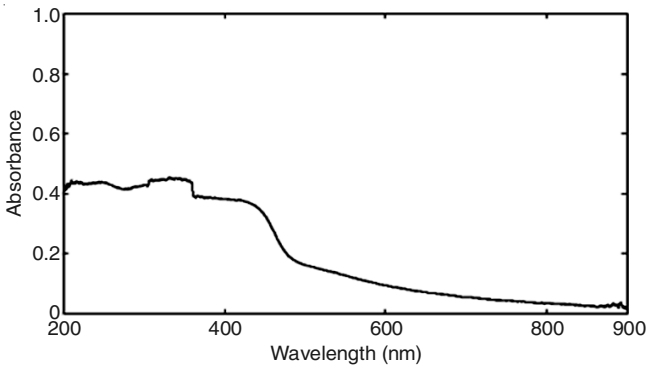

Fig. 1a. UV-visible spectrum of salicylalchitosan

Synthesized CONPs are known in the solution by the colour changing from bluish green to pale green due to the reduction of copper and from pale green to dark brown due to capping of stabilizing agent, curcumin which indicates that the formation of well reduced and stabilized CONPs. The absorption spectra of CONPs exhibit a band at around 248 nm corresponding to the absorption of copper oxide nanoparticles and the other small broad peak observed at 585 nm also indicates copper oxide nanoparticles. However, these differences may be attributed to the differences in the methods used (chemical or biological) for the synthesis of the nanoparticles as reported by Abboud *et al*. [25] and Phuo and Chyu [26]. The important another one peak observed at 415 nm could be assigned to curcumin (Fig. 1b).

Fig. 1c shows the salicylalchitosan functionalized copper oxide nanoparticles which gives the sharp band at 226 nm belongs to copper oxide nanoparticles interacted with salicylalchitosan and the important band arises at 306 nm which could be assigned to azomethine compound of salicylalchitosan. The final and broad peak observed at 401 nm which could be chitosan molecule present in the system.

FT-IR spectra studies: FTIR spectra of salicylalchitosan is shown in Fig. 2a. In the FTIR spectrum of salicylalchitosan, the following significant bands were observed. The axial vibration of O-H was observed at 3379.11 cm^{-1}, the C-H stretching band was observed at 2884.67 cm^{-1} and the important sharp peak arrived at 1626.15 cm^{-1} which can be assigned to azomethine group. The new bands arised at 1274.18 cm^{-1} and 1219.45 cm^{-1} due to the presence of phenolic C=O stretching and phenolic O-H stretching respectively.

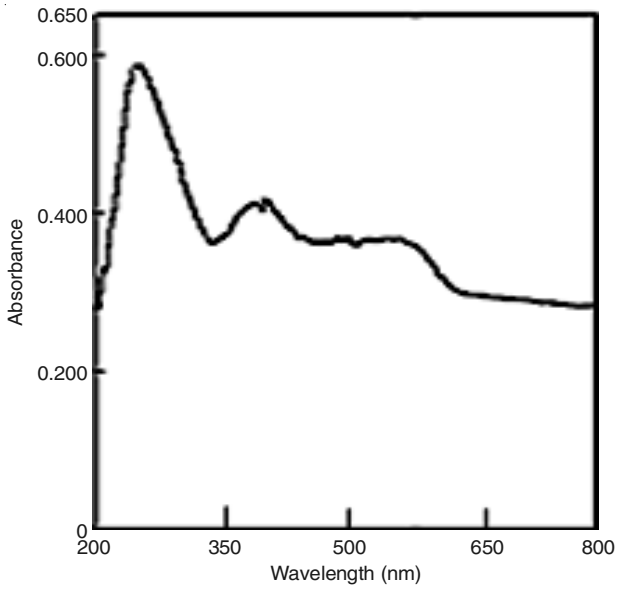

Fig. 1b. UV-visible spectrum of CONPs

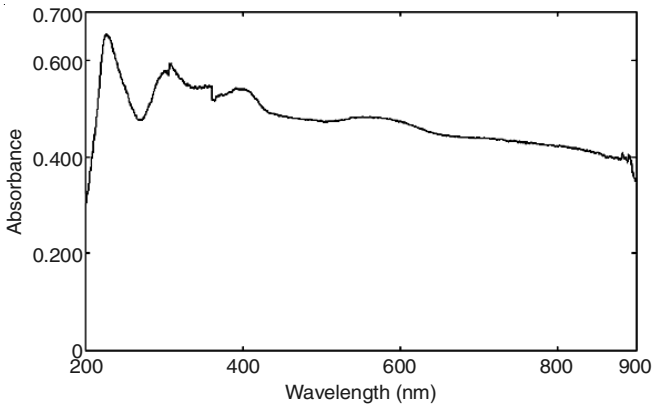

Fig. 1c. UV-visible spectrum of BCONPs

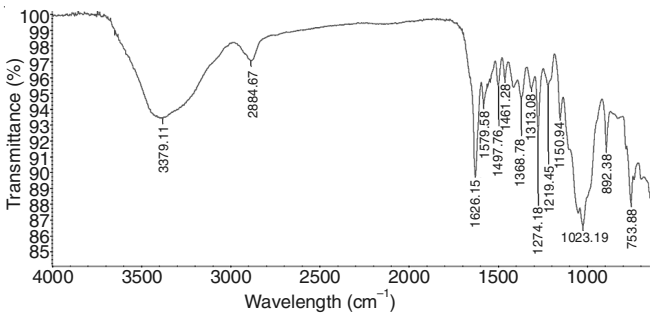

Fig. 2a. FT-IRspectrum of salicylalchitosan

Fig. 2b shows the FT-IR spectrum of copper oxide nanoparticles [27]. The phenolic OH showed its weak broad band in the range of 3342 cm^{-1} which is assigned to phenolic-OH group of curcumin moiety. The peak observed at 2913 cm^{-1} can be assigned to the –OH stretching of water or ethanol present in the system. The C=O stretching of curcumin at 1625 cm^{-1} was shifted to a higher wave number at 1700 cm^{-1} due to interaction with copper nanoparticles. Three characteristic peaks in the range of 1520–1350 cm^{-1} confirms the aromatic unsaturation (C=C) of stabilized curcumin system. The (C-O) band presence was assigned by the peaks found at 1250-1000 cm^{-1}.

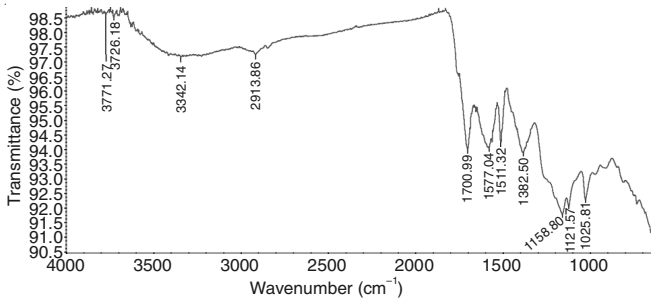

Fig. 2b. FT-IR spectrum of CONPs

Fig. 2c shows the FT-IR spectrum of functionalized copper oxide nanoparticles. From the data obtained, the broad band obtained at 3384 cm^{-1} was assigned to polymeric stretching of hydroxyl group (O-H) present in the chitosan moiety. The peak observed at 2927 cm^{-1} can be assigned to the C-H stretching of water or ethanol present in the system. The broad band exhibited at 2542 cm^{-1} belongs to the copper oxide which was functionalized with bioactive salicylalchitosan by exhibiting another band at 1943 cm^{-1} due to the presence of aromatic combination. The C=O stretching of salicylalchitosan system was confirmed by the peak raised at 1703 cm^{-1}. The two bands obtained at 1574 cm^{-1} and 1557 cm^{-1} are assigned to C=C aromatic ring stretching. The low intensity peak observed at 1385 $cm^{-1}$was assigned to phenolic OH stretching of salicylaldehyde group coordinated with the chitosan molecule. The sharp peak observed at 1179 cm^{-1} was due to the C-N stretching of tertiary amine of chitosan and the small hump observed at 1032 cm^{-1} was due to the C-N stretching of azomethine group present in the salicylalchitosan was utilized for stabilization of copper oxide nanoparticles. The (C-O) band presence was assigned by the peaks found at 900-800 cm^{-1}.

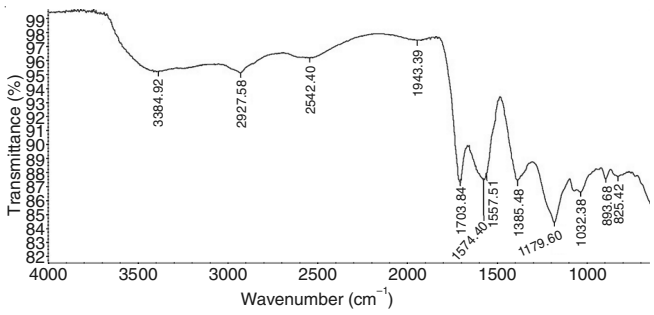

Fig. 2c. FT-IR spectrum of BCONPs

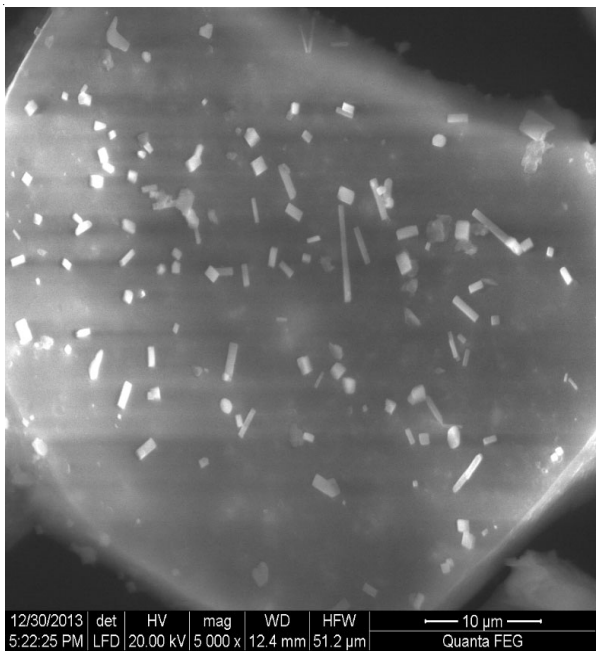

Fig. 3a. SEM image of CONPs

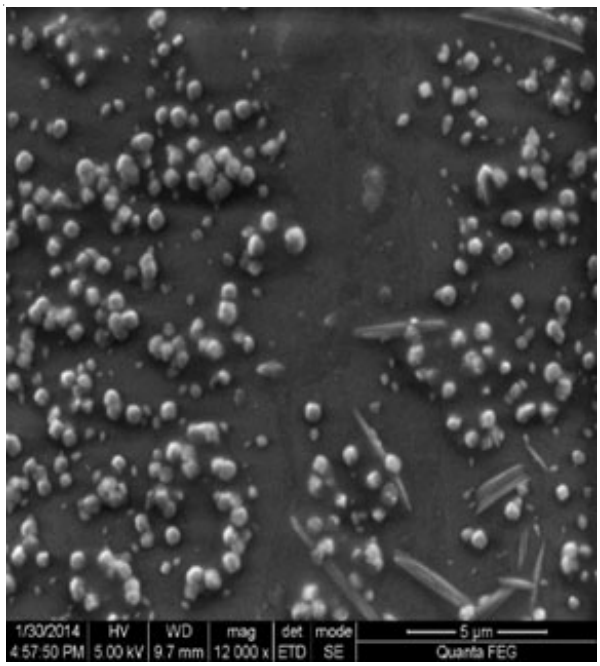

Fig. 3b. SEM image of BCONPs

SEM studies: Morphology of synthesized copper oxide nanoparticles was characterized by SEM analysis. The samples were placed in an evacuated chamber and scanned in a controlled pattern by an electron beam. Interaction of the electron beam with the specimen produces a variety of physical phenomenon that detected, were used to form images and provide information about the specimens. The SEM images of copper oxide nanoparticles are shown in Fig. 3a. It can be view that the CONPs formed are well dispersed and evenly distributed in all direction. SEM images of those compounds had shown very clear that most of the particles are cubic and rod shaped morphology of material. Fig. 3b shows SEM image of salicylalchitosan functionalized copper oxide nanoparticles. The image exhibits a mixing of dot shaped and rod shaped morphology with slight agglomeration due to the nanoparticles oxidation.

TEM studies: Fig. 4a shows the TEM image of the copper oxide nanoparticles. This image shows that the particles formed are of nearly spherical morphology. The nanoparticles are moderately dispersed and the average crystallite size of particles in the range of 60 to 100 nm.

Fig. 4b exhibits the TEM image of biofunctionalized copper oxide nanoparticles. It can be view that the BCONPs formed are well dispersed spherical morphology and the average crystallite size of particles in the range of around 100 nm. Therefore, the copper oxide nanoparticles are well formed and observed in appreciable nanosized.

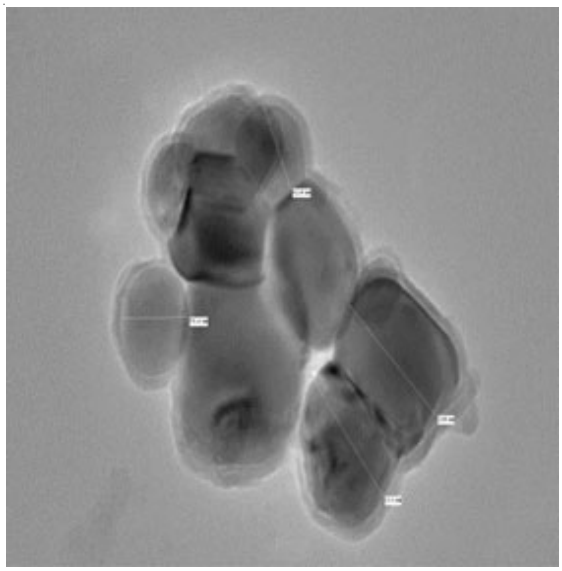

Fig. 4a. TEM image of CONPs

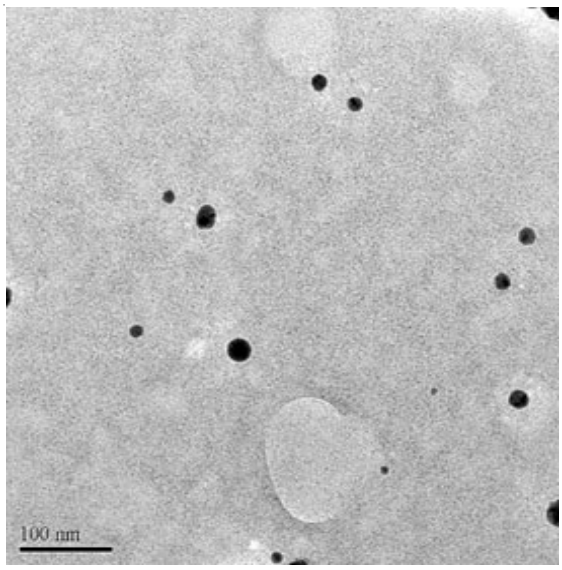

Fig. 4b. TEM image of BCONPs

Antibacterial activity: The antibacterial activities of salicylalchitosan, CONPs and salicylalchitosan functionalized CONPs against two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative bacteria (*Escherichia coli* and *Salmonella typhi*) were evaluated and their activity was compared to chloramphenicol. The results are reported in Table-1.

TABLE-1
EVALUATION OF ANTIBACTERIAL ACTIVITY

Bacterial species	Zone of inhibition diameter (mm sample^{-1})			
	C	SC	CONPs	BCONPs
S. aureus	21	22	17	23
B. subtilis	18	19	21	18
E. coli	20	17	19	14
S. typhi	19	18	18	20

C = Standard drug (chloramphenicol); SC = Salicylalchitosan; CONPs = Copper oxide nanoparticles; BCONPs = Biofunctionalized copper oxide nanoparticles

Antifungal activity: Salicylalchitosan, CONPs and biofunctonalized copper oxide nanoparticles were determined for their antifungal activity against four fungal strains *Candida albicans*, *Curvularia lunata*, *Aspergillus niger* and *Trichophyton simii* and their activity was compared with standard antifungal drug fluconazole. The results were shown in the Table-2.

TABLE-2
EVALUATION OF ANTIFUNGAL ACTIVITY

Bacterial species	Zone of inhibition diameter (mm sample^{-1})			
	C	SC	CONPs	BCONPs
C. albicans	19	18	21	11
C. lunata	19	16	16	19
A. niger	20	17	19	6
T. simii	21	20	15	11

C = Standard drug (fluconazole); SC = Salicylalchitosan; CONPs = Copper oxide nanoparticles; BCONPs = Biofunctionalized copper oxide nanoparticles

Conclusion

In summary, we have synthesized bioactive salicylalchitosan functionalized copper oxide nanoparticles by reacting salicylalchitosan derived from chitosan with copper oxide nanoparticles. Copper metal was reduced by utilizing lemon extract and stabilized by curcumin. The entire synthesis process was carried out through green synthesis manner. Synthesized copper oxide nanoparticles and biofunctionalized nanoparticles morphology studies were investigated by SEM and TEM analysis. The morphology studies revealed that the particle size of CONPs was upto 60 nm and functionalized nanoparticles was 100 nm with spherical and dot shaped morphology. The antimicrobial activity study was undertaken against two Gram-positive bacteria and two Gram-negative bacteria and four funguses. From the inhibition zone results, synthesized CONPs were showed moderate inhibition activity against over all species while the biofunctionalized nanoparticles were shown better activity than the non-functionalized nanoparticles as well as standard drug against *S. aureus*, *B. subtilis, C. lunata* and *A. niger* species. Thus our findings report that salicylalchitosan functionalized copper oxide nanoparticles synthesized from the above proposed green method is shown appreciable results in the view of pharmaceutical applications.

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