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ORIGINAL RESEARCH

Preparation and characterization of ascorbic acid-mediated chitosan–copper oxide nanocomposite for anti-microbial, sporicidal and biofilm-inhibitory activity

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Abstract

The chitosan–copper oxide (Chi–CuO) biopolymer nanocomposites were synthesized by a simple green chemistry method using ascorbic acid as a reducing and capping agent. The intense peak around 300 nm was observed in the UV–visible spectrum indicating the formation of CuO nanoparticles. The prepared Chi–CuO nanocomposites were characterized using energy-dispersive X-ray spectroscopy (EDX), scanning electron microscopy (SEM), X-ray diffraction spectroscopy (XRD), and Fourier transform-infrared spectroscopy (FT-IR). SEM and XRD pattern showed cubic shape for Chi–CuO nanocomposites with average crystalline size of 17 nm, as calculated using Debye–Scherrer's formula. The FT-IR spectral studies showed the Cu–O bond formation with chitosan to form nanocomposites. Synthesized nanocomposites showed significant anti-microbial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Penicillium notatum*, assayed using the agar well diffusion method. It also showed sporicidal activity against *B. subtilis* and exhibited effective biofilm-inhibitory activity against *B. subtilis* (69%/100 µg/mL) and *P. aeruginosa* (63%/100 µg/mL).

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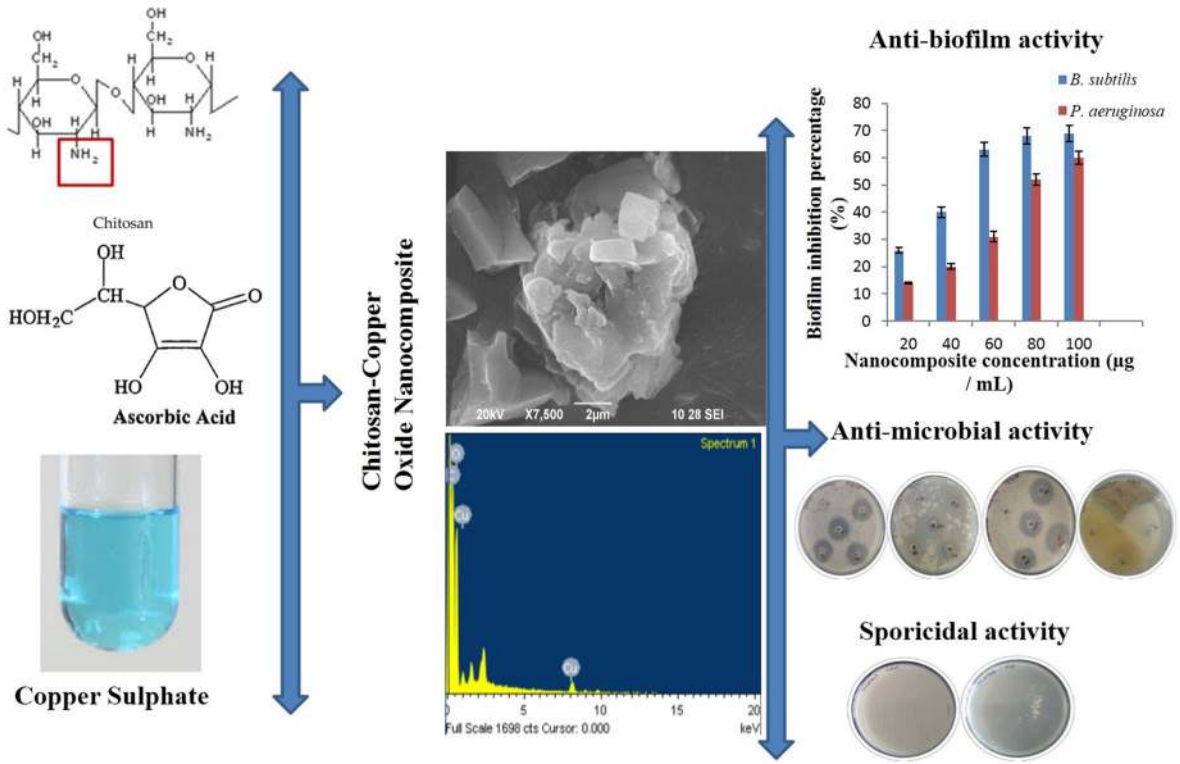
Graphical abstract



Keywords Biopolymer nanocomposites · Chitosan · Copper oxide · Anti-microbial activity · Biofilm inhibition activity

Introduction

Green synthesis of metal and metal oxide nanoparticles has attracted much attention than other nanoparticles synthesis methods such as microwave assist [1], phase transfer [2], electrochemical [3], sol–gel method and photochemical methods [4]. Green synthesis of metal and metal oxide nanoparticles using plants, microorganisms and bio-compounds is environmental friendly without using toxic compounds and involves simple preparation methods [5].

Nowadays, anti-microbial-resistant pathogens have been increasing with great frequency. Traditional medicine and some of the therapeutic drugs could not control the infections from microbial pathogens [6]. In recent years, metal oxide nanoparticles have gained attention for treating and preventing microbial pathogens. Among the metal oxide nanoparticles, copper oxide nanoparticle (CuO) capped with biologically active molecules such as carbohydrates, vitamins and proteins are the new hope as nanoparticle agents to treat microbial pathogens [7].

The importance of polymers such as chitosan and chitin on metal oxide nanoparticles has attracted attention in the pharmaceutical and biological field [8]. Chitosan is a linear polysaccharide composed of randomly distributed β-(1 → 4)-linked D-glucosamine and *N*-acetyl-D-glucosamine (Fig. 1) with a number of commercial and biomedical uses [9]. Recently, chitosan-coated polypropylene films exhibited important antibacterial activity [10]. As chitosan is in a solid form, only organisms in direct contact with the active sites of chitosan are inhibited. The CuO nanoparticles enhance

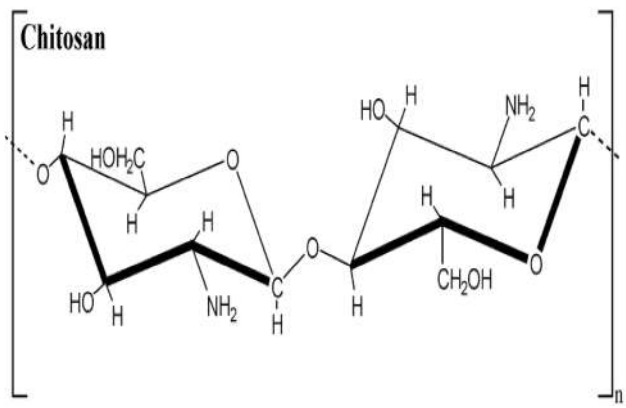


Fig. 1 Structure of chitosan biopolymer molecule

the diffusivity of chitosan, so that it can be used as an effective anti-microbial agent [11]. The anti-microbial activity of chitosan may be enhanced with incorporation of CuO into chitosan as nanocomposite. In the present study, we prepared chitosan-coated copper oxide nanocomposites using ascorbic acid as a bio-template for the first time. Here, we have demonstrated the preparation, physical characterization of Chi–CuO nanocomposites and its anti-microbial activity against bacterial and fungal pathogens, sporicidal and biofilm inhibition activity.

Materials and methods

Materials

Copper sulfate ($CuSO_4 \cdot 5H_2O$), ascorbic acid and acetic acid were purchased from Himedia Laboratories, Mumbai, India. Chitosan (medium molecular weight with 90% deacetylation) was obtained from Sigma-Aldrich. All other chemicals and reagents were of analytical grade.

Preparation of Chi–CuO nanocomposites

About 0.2 g chitosan in 10 mL of 0.04% acetic acid was mixed with 90 mL of copper sulfate solution (10 mM) containing 1% ascorbic acid and subjected to sonication for 30 min. After sonication, the prepared solution mixture was then heated at 80 for 1 h using a heating mantle. This mixture resulted in yielding a brownish-black color precipitate. The obtained precipitate was filtered through a filter paper and dried at 100 °C overnight and then samples were dried and used for further studies.

Characterization of Chi–CuO nanocomposites

The formation of CuO and Chi–CuO nanocomposites was confirmed using UV–visible spectroscopy (JASCO-V-670). UV absorbance spectral analysis was studied in the range from 200 to 500 nm at a resolution of 2 nm. The surface morphology of Chi–CuO nanocomposites was observed using scanning electron microscopy (SEM, Model-JEOL JSM-6400). The presence of elements was identified through energy-dispersive X-ray spectroscopy (EDX) attached with SEM. X-ray diffraction (XRD) analysis was carried out to determine the crystalline structure of the prepared Chi–CuO. X-ray diffraction was recorded in the 2θ range from 10 to 90 at 40 kV/40 mA current with CuKa radiation (SHIMADZU, XRD-7000). The presence of bio and polymer functional groups that are responsible for the reduction, capping and formation of Chi–CuO samples was studied by FT-IR (SHIMADZU, Prestige 20 IR-Spectrometer).

Anti-microbial activity

The biocidal activity of Chi–CuO nanocomposite was investigated against Gram-positive (*Bacillus subtilis* ATCC 6633) and Gram-negative (*Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 443) bacterial strains and a fungus (*Penicillium notatum*), using the well diffusion method as described by Naika et al. [12]. Bacterial and fungal strains were swabbed on nutrient and potato dextrose agar plates, respectively, using cotton swabs. The required wells were made on agar plates with the help of gel puncture, after which it was impregnated with different concentrations of Chi–CuO (25, 50, 100 µg/mL) and gentamicin (100 µg/mL) was used as control; it was then incubated for 24 h to observe the zones.

Biofilm inhibition activity

The biofilm inhibition activity of Chi–CuO nanocomposites was determined using a method described by Bharathi et al. [13]. Each well of the sterile microtiter plate (96 wells) was filled with 90 µL of Muller Hinton broth and inoculated with 10 µL of test bacterial pathogens (*B. subtilis* and *P. aeruginosa)*. To the above mixture, different concentrations of Chi–CuO composites (20, 40, 60, 80 and 100 µg/mL) were added and incubated for 24 h at 37 °C. The medium of each well was removed and excess adherent cells were washed with 0.3 mL of saline buffer to remove floating bacteria. Each well was stained with 0.4% crystal violet, and the excess stain washed using sterile water. After drying for 15 min, the stains were solubilized with 100 µL of 70% ethanol and read at 595 nm using an ELISA reader (BioRad-680, USA). The inhibitory percentage of biofilm formation was calculated as done by Bharathi et al. [13] and Barapatre et al. [14].

In vitro sporicidal activity

Bacillus subtilis culture was grown for 1 week at 37 °C on nutrient agar. Plates were scraped and the culture was suspended in 50% ethanol. The suspended culture was then incubated at 22 °C for 2 h with agitation to lyse the vegetative bacteria. The suspension was centrifuged at 8000 rpm for 20 min and the pellet was washed twice in cold distilled water. The spore pellet was resuspended in nutrient broth and used immediately for experiments. The typical digestive

agar was autoclaved and cooled to 55 °C. About 250 µg/mL of Chi–CuO nanocomposites was added into 25 mL of agar and continuously stirred while the plates were poured. About 10 µL of spore preparation was plated. The plates were incubated for 24 h aerobically at 37 °C and evaluated for growth.

Results and discussion

Synthesis of Chi–CuO nanocomposites

Initially, the synthesis of Chi–CuO nanocomposites was visually verified by the color change from blue to green and then finally brownish-black color precipitate. The color change may be due to the reaction with copper sulfate, ascorbic acid and chitosan compounds. Ascorbic acid may act as a reducing and stabilizing agent for the formation of CuO nanocomposites. Similar to our report, Sutradhar et al. [15] visualized color change of copper oxide nanoparticles from light orange to brownish-black color when adding coffee powder extract to copper salt.

Characterization of Chi–CuO nanocomposites

The CuO peak was formed around 300 nm (Fig. 2a). The formation of Chi–CuO nanocomposites was monitored by UV–visible spectroscopy and exhibited broad absorbance peak ranging from 270 to 310 nm due to its surface plasmon resonance (SPR). A maximum was attained at 300 nm (Fig. 2b) and was in accordance with others' reports [16].

Figure 3a–c shows the morphology of synthesized Chi–CuO nanocomposites. Figure 3a shows the prepared nanocomposites were cubical structure with varying size and Fig. 3b, c shows the irregular shape with the cubical aggregated structure of the prepared Chi–CuO nanocomposites. Similar to our study, copper oxide nanoparticles exhibited a cubic structure, synthesized using different capping agents [17]. The EDX spectrum analysis represented in Fig. 3d shows the signal from Cu together with C and O, and the elemental composition is represented in Table 1. Thus, the formation of CuO nanoparticles is confirmed, and Cu is the main element in synthesized nanoparticles. Other absorbance peaks of O and C in the spectrum obtained were mostly due to the emission of X-ray from existing O- and C-related compounds of ascorbic acid and chitosan [5].

Figure 4 shows the XRD diffraction pattern of the prepared Chi–CuO nanocomposites. Diffraction peaks at 2θ of 12° and 20° correspond to (020), (220), which showed that the chitosan had coated on copper oxide. The spectrum also shows the peaks in the range of 22.9°, 25.5°, 32.2°, 45.6°, 53.1°, 56.2°, 65.9°, 69.1°, and 77.4°, which correspond to the (021), (021), (110), (111), (020), (202), (− 113), (202) and (111) planes, respectively [18]. The XRD spectrum revealed that the prepared Chi–CuO nanocomposites had cubic crystalline structure, and the corresponding planes obtained were matched with the JCPDS File number (JCPDS NO: 05-0061) [18, 19]. The average crystalline size was calculated using Debye–Scherrer's formula:

$$D = 0.9\lambda/\beta \cos \theta,$$

where D is the average size of the nanoparticles, λ is the wavelength of X-ray, β is the FWHM (full width at half maximum) in radians and θ is the diffraction angle [13]. The average mean crystallite size was found to be 17 nm from all breath of the refraction.

The FT-IR spectra of the prepared Chi–CuO nanocomposites, chitosan and ascorbic acid are shown in Figs. 5, 6

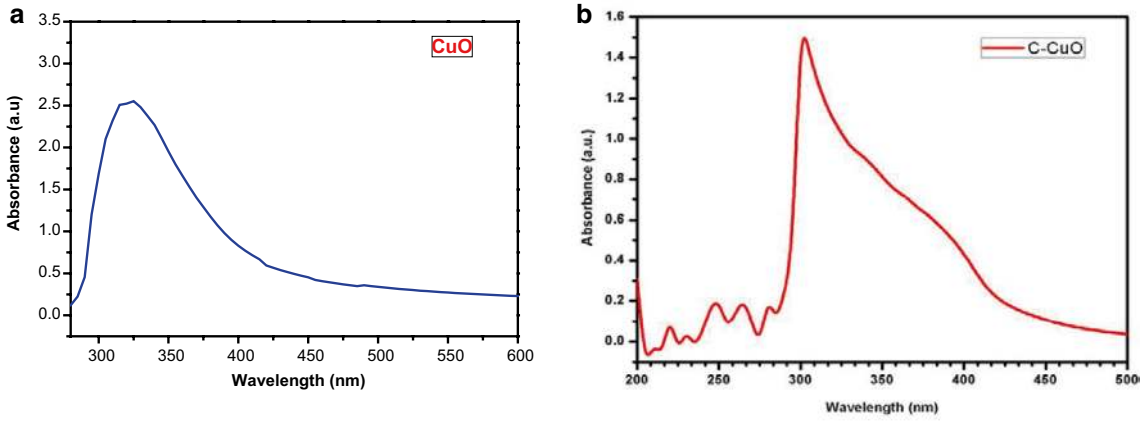


Fig. 2 UV–visible spectral absorbance of (**a**) CuO and (**b**) Chi–CuO nanocomposites

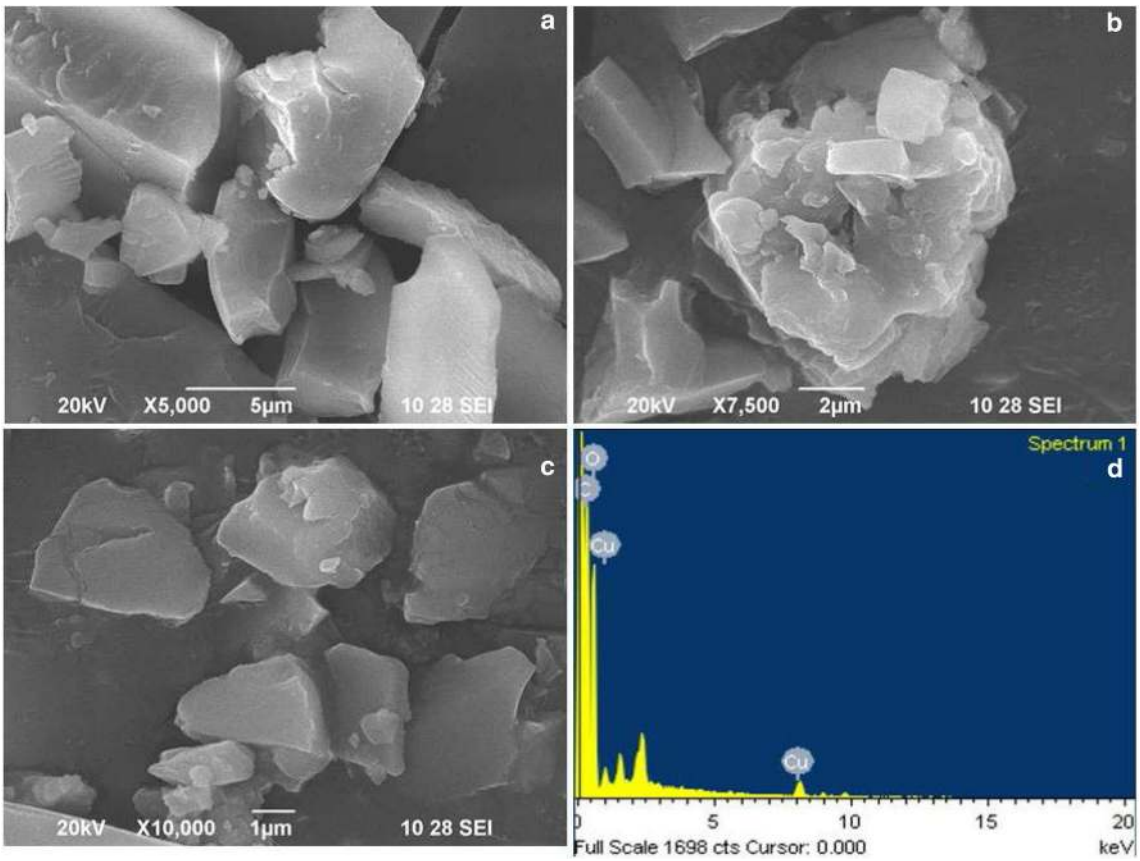


Fig. 3 Electron microscopic observation and elemental analysis of Chi–CuO nanocomposites, (**a**–**c**) SEM image of Chi–CuO nanocomposites and (**d**) EDX spectrum of Chi–CuO

Table 1 Elemental composition of chitosan–CuO nanocomposites

Element	Weight %
C	45.70
O	50.54
Cu	3.77
Total	100.0

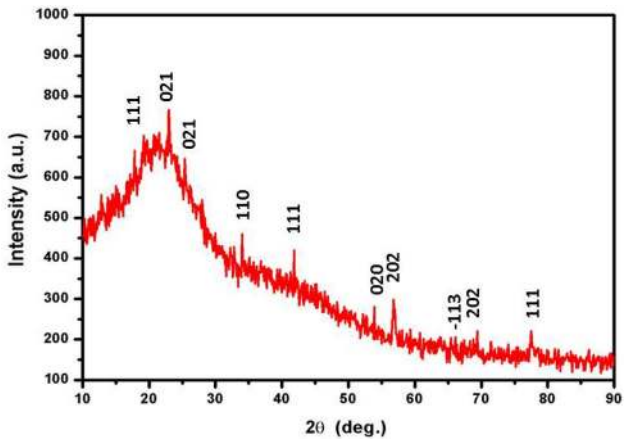


Fig. 4 XRD pattern of the prepared chitosan–copper oxide nanocomposites

and 7. The prepared Chi–CuO exhibited a strong intensity peak at 3577.14 cm^{-1}, assigned to alkyl (O–H) stretch possibly of alcohols and phenols. Medium peaks at 1711.90 and 1585.55 cm^{-1} corresponded to the C=O stretch and C–C vibrations of carboxylic acids and aromatic compounds. Peaks at 1377.23 and 1069.57 cm^{-1} assigned to C–H and C–N stretch indicated the presence of alkanes and aliphatic amines (chitosan). Low-intensity peaks near 800–400 cm^{-1} represent the presence of metal oxygen [20–22]. FT-IR spectrum from Figs. 6 and 7 clearly shows that the major spectral peaks of the prepared Chi–CuO composites are derived from ascorbic acid and chitosan. The presence of these bio and polymer compounds may be responsible for the reduction and conversion of synthesized metal nanocomposites (Chi–CuO) [5].

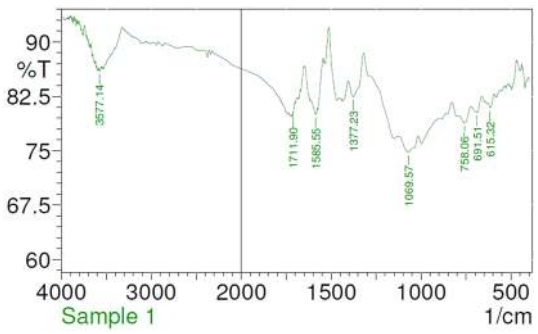


Fig. 5 FT-IR spectrum of the prepared chitosan–copper oxide nanocomposites

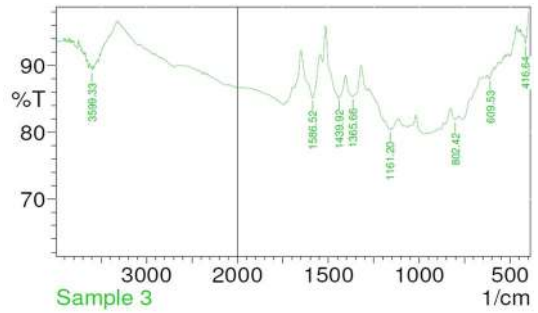


Fig. 6 FT-IR spectrum of chitosan biopolymer

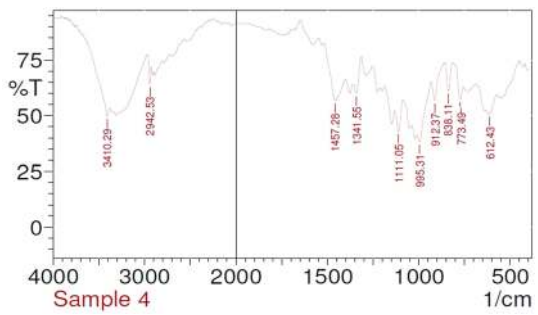


Fig. 7 FT-IR spectrum of ascorbic acid

Anti-microbial activity

The anti-microbial activity of Chi–CuO composites were examined against bacterial and fungal strains using the agar well diffusion method. The highest antibacterial zone of inhibition was recorded against *B. subtilis,* followed by *P. aeruginosa*, *E. coli* and *P. notatum* (Fig. 8a–d). Measurement of the inhibitory concentration is shown in Table 2. Increasing concentrations of Chi–CuO nanocomposites exhibited increasing activity. The anti-microbial activity of the prepared Chi–CuO nanocomposites was similar to that of the control gentamicin (100 µg/mL). The anti-microbial activity mainly depends on the size and shape of the copper oxide nanoparticles [23]. Polymer-coated copper oxide nanocomposites showed potent antibacterial activity compared to CuO nanocomposites [24]. Chitosan has strong affinity toward metal ions due to the presence of many amine and hydroxyl groups, and thus leading to Chi–CuO nanocomposite formation [25]. Ascorbic acid act as a reducing and capping agent to impart stability to the nanocomposite [26]. The anti-microbial property of metal/polymer nanocomposites may be due to the adsorption of bacteria and causes disruption of the membrane [27]. Similar to our results, the antibacterial activity of hybrid chitosan–cerium oxide nanoparticles was recorded against *E. coli* and *B. subtilis* [28]. Chitosan nanoparticles have biodegradability, biocompatibility, stability and low toxicity and can act as a novel drug carrier [29].

Anti-biofilm activity

The biofilm inhibition activity of Chi–CuO nanocomposites was assayed on *B. subtilis* and *P. aeruginosa* using microtiter plate assays. It was observed that the biofilm inhibition activity of Chi–CuO increased in a concentration-dependent manner (Fig. 9). Similar to our study, copper oxide nanocomposites synthesized from medicinal plants showed increased percentage of anti-biofilm activity with the increasing concentration of CuO [30]. Treatment for 24 h with 100 µg/mL of Chi–CuO composites showed a decrease of 69 and 63% of the biofilms formed by *B. subtilis* and *P. aeruginosa*. These results demonstrate that synthesized Chi–CuO nanocomposites induce detachment of *B. subtilis* and *P. aeruginosa* biofilms, with efficient concentration of the prepared nanocomposites. Similar to our results, Kaliswarlal et al. [31] also reported the inhibition of biofilm formation, irrespective of the species tested.

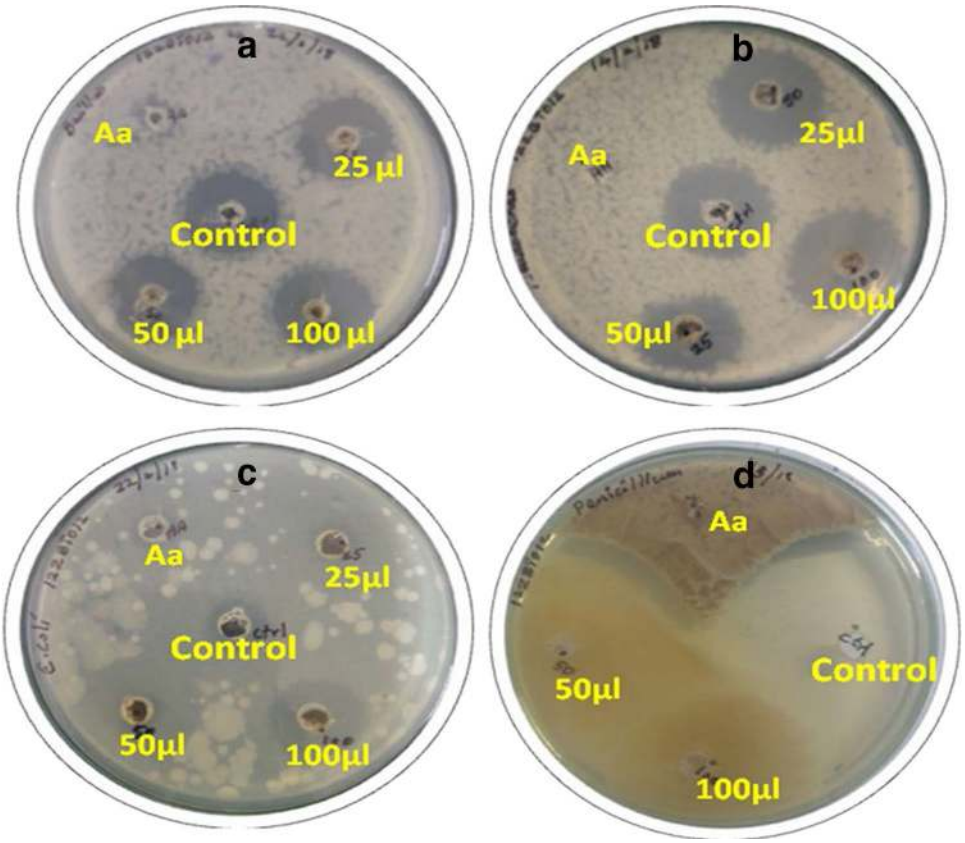


Fig. 8 Anti-microbial activity of C–CuO nanocomposites against (**a**) *B. subtilis*, (**b**) *P. aeruginosa*, (**c**) *E. coli* and (**d**) *P. notatum* with different concentrations (*Aa* ascorbic acid; control, gentamicin)

Table 2 Anti-microbial activity of Chi–CuO nanocomposites showing the zone of inhibition

Species	Zone of inhibition (mm)			
	Gentamicin	25 µL	50 µL	100 µL
B. subtilis	9 ± 0.5	6.5 ± 0.5	7 ± 0.5	8 ± 0.5
E. coli	9 ± 0.5	6 ± 0.5	6 ± 0.5	8 ± 0.5
P. aeruginosa	8 ± 0.5	6 ± 0.5	6.5 ± 0.5	8 ± 0.5
P. notatum	11 ± 0.5		10 ± 0.5	10 ± 0.5

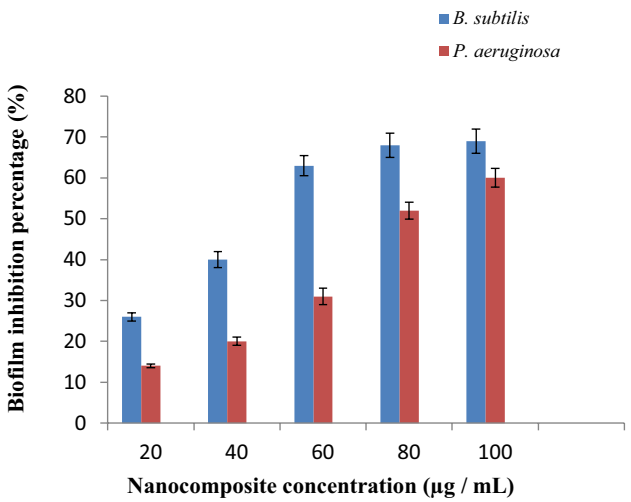


Fig. 9 Biofilm inhibition percentage of the prepared C–CuO nanocomposites

Sporicidal activity

Ascorbic acid-mediated Chi–CuO nanocomposites showed sporicidal activity by effectively killing 98% *B. subtilis* spores. The control plate contains only the medium and the diluted spores of *B. subtilis* which have uncountable colonies, whereas the nanocomposite inhibits the growth of the spores and the growth is countable. The sporicidal activity is shown in Fig. 10. Overall, eco-friendly prepared cubical-shaped Chi–CuO nanocomposites exhibited good anti-microbial, sporicidal and anti-biofilm activity similar to other researchers [32].

Conclusion

CuO polymer nanocomposites were synthesized using ascorbic acid as reducing agent and then chitosan was coated on prepared CuO solutions. The prepared Chi–CuO nanocomposites were cubical shaped with an average size of ~ 17 nm as determined by SEM and XRD. The prepared nanocomposites showed significant antibacterial activity and potent anti-fungal activity. Furthermore, Chi–CuO nanocomposites showed sporicidal activity against *B. subtilis* spores'

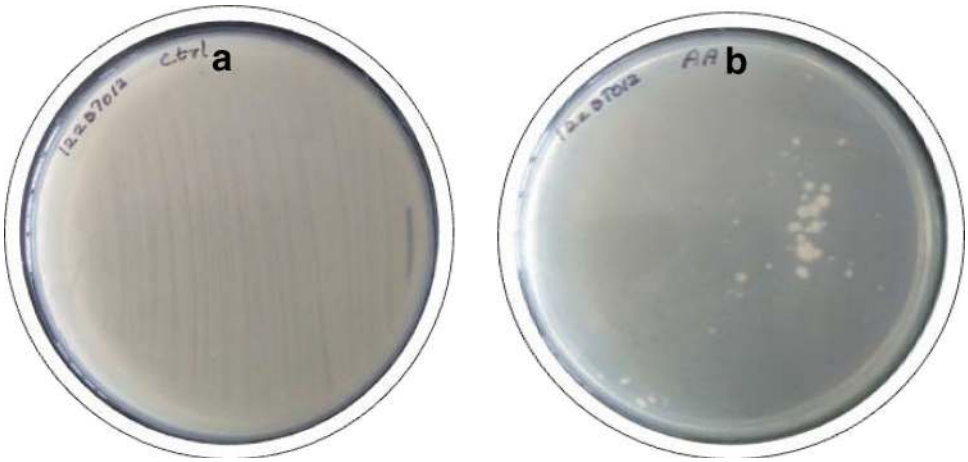


Fig. 10 Sporicidal activity of Chi–CuO nanocomposites against *B. subtilis* spores: (**a**) control and (**b**) Chi–CuO NPs

potential biofilm inhibition activity against *B. subtilis* and *P. aeruginosa*.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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