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"Nano-Copper": A Potential Remediation of Antibiotic-Resistant Infections

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Abstract

Use of copper to cater various needs of human civilization started immediately after the neolithic age. However, the nano-formulation of copper containing copper or one of its compounds including oxides in order to combat some of the societal challenges are quite recent. Here we have synthesized copper oxide nano particles and functionalized with acetate ligands to form a nano-hybrid which is shown to have medicinal properties. We have used electron microscopy, X-ray diffraction and dynamic light scattering tools for the structural characterization of the nano-hybrid. Functionalization of the copper oxide nanoparticles has been confirmed by FTIR and UV-Vis spectroscopy studies. A detailed study on the functionality of the nano-hybrid is shown to be very promising for antibiotic resistant bacterial infection remediation which is the need of the hour. Light activation enhances the antibacterial efficacy manifold making the nano-hybrid suitable for applications like photodynamic therapy. We have also used computational biology strategy in order to rationalize the antibiotic resistant bacterial remediation (particularly MRSA strains) found in our experimental studies.

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Introduction

Copper oxide nanoparticles (CuO NPs) have attracted high degree of research interests over other metallic nanoparticles by virtue of their tremendous potential applications in diverse fields like chemo and biosensing, medicinal therapeutics, particularly as anti-microbial, anti-bacterial and anticarcinogenic agents, drug delivery systems, energy saving devices, optics and optoelectronics, electrochemistry and catalysis [1-9]. However, CuO, the semiconducting compound with a monoclinic structure is mainly considered most for its well reported antimicrobial properties [10]. It is used widely as an antifungal, antibiotic and antimicrobial agent when introduced into textiles and coatings [11] but limited information on the antimicrobial activity of surface functionalized CuO is available. Further surface modifications of CuO NPs by post-functionalization approach introduces unique physical and chemical properties [12,13] including possible enhancement of their antimicrobial activities.

Antimicrobial-Resistant (AMR) infections are declared as one of the top ten global public health threats by World Health Organization (WHO) and it was reported that in the year 2019 only, the AMR organisms were the primary cause of 1.27 million deaths worldwide [14]. Amongst AMR bacteria, Multi Drug Resistant (MDR) species are of greater concern because of their capability to mutate genes and developing resistance towards antibiotics. Methicillin-resistant Staphylococcus aureus (MRSA) is such a notorious MDR bacteria that has been categorized as a high priority multidrug-resistant pathogen by WHO [14]. Improper and uncontrolled use of antibiotics is the root cause of rise in bacterial resistance, making the clinical management of infections harder to manage using conventional antibiotics. MRSA is resistant to many antibiotics like penicillin, methicillin, oxacillin and amoxicillin [15]. The sustained emergence and rapid spread of MRSA infections along with the scarcity of new antibiotics provoke urgent interest in alternative and novel antimicrobial agents such as small antibiotics, metal NPs, cationic polymers and antimicrobial peptides [16-20].

In the current study, we reported synthesis, characterization and antimicrobial activity of acetate functionalized CuO NPs on MRSA bacterial strain. CuO NPs were synthesized and capped by precipitation technique [21] and grafting method respectively [22]. Structural properties of CuO NPs were examined by X-Ray Diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM) equipped with Energy Dispersive X-Ray Spectroscopy (EDS). Dynamic Light Scattering (DLS) and zeta potential studies were also employed for estimating the hydrodynamic diameter and solubility assessment of the synthesized nano hybrid. Functionalization of CuO NPs by acetate ligands were confirmed by FTIR and UV-Vis spectroscopy. Antimicrobial activity of acetate-CuO NPs was examined on MRSA bacteria strain. The acetate functionalized NPs were found to produce reactive oxygen species (ROS) upon photo-excitation, which is responsible for their anti-microbial action as ROS has the ability to destroy the active substances in the bacterial inner and outer membrane [23-25]. This phenomenon establishes the credentials of acetate CuO NPs for applications like antibacterial Photo Dynamic Therapy (PDT) with enhanced efficacy. We have also used computational biology strategy in order to rationalize the antibiotic resistant bacterial remediation found in our experimental studies.

Materials and methods

Materials

All the chemicals were analytical grade and used as procured without any further purification. Copper acetate, glacial acetic acid, sodium hydroxide and Ethanol were purchased from Sigma Aldrich (St. Louis, MO, USA), California. 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was bought from Calbiochem to extend our study for production of ROS under white light irradiation. For bacterial studies, LB top agar and Luria Broth (LB) medium were bought from HIMEDIA. Pure Millipore water was used during all the experiments. Methicillin-Resistant Staphylococcus Aureus (MRSA) strain (ATCC 25923) was procured from ATCC.

Synthesis of functionalized CuO nanoparticles:

The synthesis was carried out following previously described methodology in Zhu et al [21]. The acetate ligands generated during the synthesis process, provided satisfactory passivation against aggregation yielding high colloidal stability. In brief, 0.54 gm copper acetate was dissolved in 150 ml of deionized water. 0.52 gm glacial acetic acid was then added and the mixture was brought to boiling at 100°C under vigorous stirring. Subsequently, 0.7 gm sodium hydroxide (0.015 mol) was quickly added until the pH value of the mixture reached 6-7, where a large amount of dark brown precipitate was simultaneously produced (Figure 1a inset). The blue-coloured mixture was observed to turn brown immediately indicating the formation of CuO NPs. After 5 more minutes of reflux, the mixture was cooled to room temperature under stirring. The CuO-NPs were then isolated by centrifugation (4000rpm, 10 minutes) and washed twice with water and twice with ethanol, respectively. The supernatant containing acetate capped CuO NPs was separated. Precipitated CuO-NPs were redispersed in deionized water for further use.

For further functionalization of the CuO NPs, acetic acid grafting method was followed. 65 mM CuO and 200 mM acetic acid suspensions were prepared in a 8:2 v/v ratio of water to ethanol. The CuO suspension was mixed with the prepared acetic acid solution and the pH of the mixture was adjusted to pH 12 by adding 6 M NaOH. The mixture was then refluxed for 3 hrs. Afterward, the product was centrifuged and washed thoroughly three times to remove excess acetic acid.

Characterization Tools and Techniques

The X-ray diffraction pattern of synthesized CuO nanoparticles was measured in a PANalytical XPERTPRO diffractometer, with Cu Kα radiation (at 40 mA and 40 kV) generating at a rate of 0.02° s^{-1} in the 2θ range from 20° to 90°. A qualitative assessment of the appearance of the synthesized NPs was performed by scanning electron microscopy. The coverslips were coated with gold and scanned in a field emission scanning electron microscope (Quanta FEG 250: source of electrons, FEG source; operational accelerating voltage, 200 V to 30 kV; resolution, 30 kV under low vacuum conditions: 3.0 nm; detectors, large field secondary electron detector for low vacuum operation). The optical absorption experiments were performed using a Shimadzu spectrophotometer (UV-2600). The room-temperature steady-state emission spectra were measured using a Fluorolog Model LFI-3751 (Horiba-Jobin Yvon, Edison, NJ) spectrofluorometer. Dynamic light scattering (DLS) and ζ potential measurements were performed using a NanoS Malvern (Zeta-seizer) instrument equipped with a 4 mW He:Ne laser (λ = 632.8 nm) and a thermostated sample chamber. We have used Fourier transform infrared spectroscopy (FTIR) of the liquid samples, and the spectra were obtained using a Vertex 70V instrument (Bruker, Germany). Quartz cuvettes with a path length of 10 mm were used to perform all spectroscopic experiments.

Quantification and Characterization of ROS

2’,7’-Dichlorofluorescein (DCFH) is a well-known reagent for the quantification of ROS generation. It was prepared from DCFH-DA via a de-esterification reaction at room temperature following a standardized protocol described in previous studies [26,27]. In the presence of light, DCFH oxidation leads to the production of DCF, which gives fluorescence [28,29]. DCFH was converted into DCF by the ROS generated in the aqueous acetate-CuO medium and DCF has a characteristic fluorescence emission maximum at 522 nm upon excitation at 488 nm. The emissions were recorded using the Fluorolog Model LFI-3751 (Horiba-Jobin Yvon, Edison, NJ) spectrofluorometer. The ROS experiments were performed in the dark for 10 mins followed by under irradiation of a white light of 400−700 nm wavelength for 30 mins.

Bacterial Strain and Culture Conditions

The antibacterial action of the synthesized samples has been studied against a strain of Methicillin-Resistant Staphylococcus Aureus (MRSA) bacteria. The MRSA strain (ATCC 25923) was procured from ATCC. For antibacterial assay, fresh MRSA bacteria have been cultured using Luria–Bertani (LB) medium in a shaker incubator at a temperature of 37 °C for 28 hrs. The freshly grown MRSA culture was further diluted 10^6 times and test samples were added. The treatment of bacteria was performed on LB agar plates by the Colony-Forming Unit (CFU) assay method under dark and white light illumination conditions. The cells were incubated with 1 mM of acetate CuO NP solution for 3 hrs with photo-activation. Then the cultures were uniformly spread on LB agar plates and the plates were incubated at 37°C for 24 hrs to get the CFUs. To quantify the antibacterial activity, the CFU numbers were manually counted and presented as a bar diagram.

Statistics

All data are represented as the mean ± standard deviation unless otherwise stated. Unpaired 2-tailed T-Test was used to calculate differences between the groups. $P < 0.05$ was considered significant. GraphPad Prism (v8.0) software was used for all statistical tests.

Method of Computational Biology

To predict the Chemical-Protein (CP) Interaction Networks of CuO NP on MRSA, the web-resource STITCH (version 5.0) provided by STITCH Consortium2016 (http://stitch.embl.de/) was used. About 9,60,0000 proteins and 4,30,000 chemicals curated from 2031 eukaryotic and prokaryotic genome can be predicted by the STITCH database [30,31]. The association for a chemical-protein interaction can be predicted by their confidence score, where a higher score corresponds to a stronger interaction. Here a medium confidence score of 0.4 was considered for the present study. Eight different sources, i.e. experiments, neighbourhood, text mining, gene fusion, databases, co-occurrence, co-expression and predictions are used to populate the active interactions.

Results and discussion

The identification of precise elemental composition, particle size range and surface morphology of the acetate functionalized nanoparticulate CuO is a prerequisite to a full understanding of its potential application capabilities. The XRD pattern of the synthesized CuO NPs is depicted in **Figure 1a**. It was determined that all CuO NPs were in a monoclinic geometry with a space group of C2/C. No characteristic peaks of any impurities were detected, suggesting that high quality of CuO NPs was prepared. Moreover, the obtained χ^2 value of 1.85 for the Le Bail fitting indicates excellent agreement with the previously reported literature [32,33]. The crystallite size has been estimated from the XRD pattern using the Debye Scherrer’s equation (1) [34]:

$$D = K\lambda/\beta \cos\theta \qquad (1)$$

Where K = 0.94 is the shape factor, λ is the X-ray wavelength of Cu Kα radiation (1.541 Å), θ is the Bragg diffraction angle and β is the Full Width At Half Maxima (FWHM) of the respective diffraction peak. The crystallite size corresponding to the highest peak observed in XRD was found to be 24.31 nm. The presence of sharp structural peaks in XRD patterns and crystallite size less than 100 nm corresponds to the nanocrystalline nature of synthesized CuO NPs. The peaks at 32.5, 35.4, 35.5, 38.7, 38.9,46.2, 48.8, 51.3, 53.4, and 56.7 in 2θ correspond to the different CuO planes, respectively [35].

Figure 1b depicts the EDAX spectrum of synthesized CuO NPs. The EDAX result shows that there are no other elemental impurities present in the prepared CuO NPs. FESEM image of acetate-CuO is shown in inset of **Figure 2b**. The synthesized nano-hybrid is seen to be consisting of clustered spherical particles of approximate diameter 38 nm. The average diameter of CuO NPs was calculated from measuring over 100 particles in random field of FESEM view.

The SEM-EDAX analysis demonstrated that the atomic composition of the Cu and O elements were 54.1% and 45.2%, respectively. The mean ratio of Cu and O was therefore 54.1:45.2 and an accurate compound formula based on this atomic ratio of Cu and O can thus be given as Cu1.2O or CuO0.8. Therefore, it can be ensured that most of the synthesized nanoparticulate sample was indeed CuO.

The attachment of acetate on the surface of the CuO nanoparticles was ensured by FTIR of the functionalized CuO nanoparticle along with acetic acid **(Figure 1c)**. The acetic acid showed an absorption band at 1700cm^{-1}, which can be attributed due to the C=O stretching of the carboxylate group of acetic acid [36]. In addition, the absorption peak occurring at 1300cm^{-1} may be attributed to the bending of the O-H group of the C-OH bond of the carboxylic acid portion [37]. Moreover, the absorption band at ~3500 cm^{-1} is due to the –OH of the acetic acid [37]. In the IR spectrum of the acetate capped CuO nanoparticle, the stretching frequency of C-O and C=O has been found at ~1360cm^{-1} and ~1780cm^{-1}. This shift in the C-O and C=O indicates that the CuO nanoparticles are functionalised by the adsorption of the carboxylate group of acetic acid on the nanoparticle surface [38,39]. Moreover, the weakening of the –OH bond in the acetate capped CuO nanoparticle, further confirms the surface functionalization of CuO with acetic acid.

The hydrodynamic diameter of the synthesized CuO nanohybrid was estimated to be 50.7 nm from the Dynamic Light Scattering (DLS) studies **(Figure 1d)**. The results of DLS corroborates with the size obtained from XRD and FESEM analysis. Additionally, the Acetate CuO NPs exhibited a ζ-potential of magnitude -18.3mV assuring moderate solubility of the NPs. This result lowers the possibility of instability and particle agglomeration or precipitate tendencies of the CuO NPs out of the solutions.

The UV-Vis spectra in **Figure 2** of the acetate capped CuO exhibited a broad absorbance peak at 285 nm characteristic of surface plasmon resonance of the CuO nanparticles [40]. A less strong peak at around 350 nm, signifies the d-d transition of CuO nanoparticles due to acetate functionalization [41].

Photoinduced ROS generation capability of the Acetate CuO NPs is illustrated using a well-known non-fluorescent probe, DCFH **(Figure 3)**. DCFH is oxidized to fluorescent dichlorofluorescein (DCF) by ROS, exhibiting an emission near 522 nm upon excitation at 488 nm. Thus, the enhancement of the ROS generation level is indicated by the increase in the emission intensity at 522 nm [24]. The oxidation of only DCFH control and CuO NPs are monitored for 10 mins in the dark and then under irradiation of white light (400-700nm) for 30 mins. In the dark, there is no considerable enhancement of emission intensity at 522 nm indicating absence of dark ROS generation. However, with the increase in the light exposure time, a greater increase of emission intensity is observed for the actetate CuO nanohybrid as compared to the control **(Figure 3)**. This confirms the ROS generation capability of the synthesized nanohybrid under light exposure making it suitable to apply for photodynamic therapy.

The antimicrobial activity of the synthesized acetate-CuO NPs was investigated against the MRSA growth to explore the antibiotic potential against bacterial infections. To probe the antibacterial action of the nanoparticles they were used for incubating the culture for 3 hrs. As shown in Figure 4a, minimal colonies were observed (the bacterial growth is found to have decreased by 78.88 % in CFU from the control plate) for the nano hybrid under white light illumination condition. The bacterial growth is found to be decreased by 8% only in CFU under dark condition. The effect of two different concentrations of copper nanohybrid under white light irradiation condition was then studied further upon the growth of MRSA bacteria (Figure 4b). In case of 10uM concentration, the bacterial growth is found to be decreased by 46% CFU. On the other hand, a huge decrement of the bacterial growth is observed for 50uM concentration of acetate CuO, 92% reduction in terms of CFU with respect to the control. From these results it is evident that the nanohybrid itself is an antibacterial agent and its efficiency enhances manyfold upon white light exposure which triggers an overall huge antibacterial effect.

To study the effect of the acetate CuO nanohybrid, bacterial cultures were performed 5 times for each group (control in dark and light, acetate CuO in dark and light, different concentrations of the nanohybrid etc.) and their difference was calculated to identify their significance level. The sample size for each of the groups were 5. The p-value was calculated using unpaired 2-tailed T-Test and p<0.05 was considered to be significant. Statistical difference in between control and treatment is designated by '*'. The '*' represents p-value<0.1 and '****' represents p-value<0.0001[42].

The extraordinary effect of the synthesized copper nanohybrid may be hypothesized using predictive biological interactions **(Figure 5)**. To grasp the mechanism of action of acetate CuO NPs on MRSA, a separate comprehensive table **(Table A)** of target proteins/compounds and their biological activities are listed below.

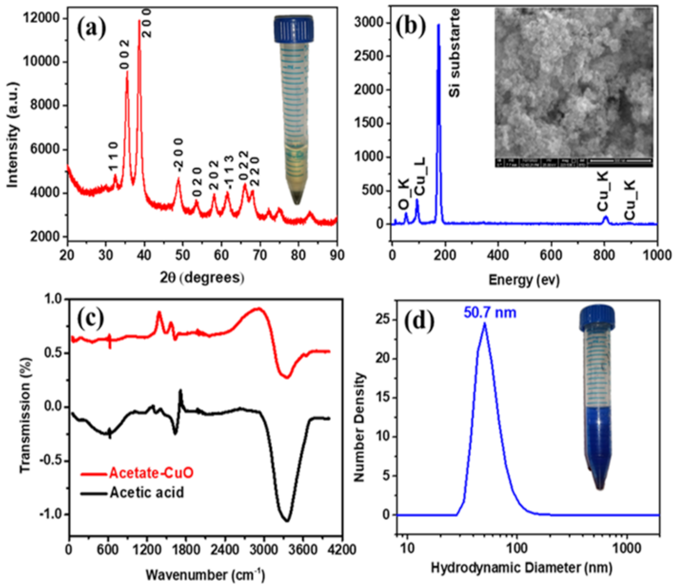

Figure 1: **(a)** XRD of the synthesized CuO nanoparticles. Inset shows the CuO nanoparticles precipitated after centrifugation during synthesis process. **(b)** FESEM image and EDAX of CuO NPs **(c)** FTIR spectra of acetate capped CuO and acetic acid in therange 0 to 4000 cm^{-1} **(d)** DLS of the acetate capped CuO from supernatant (inset).

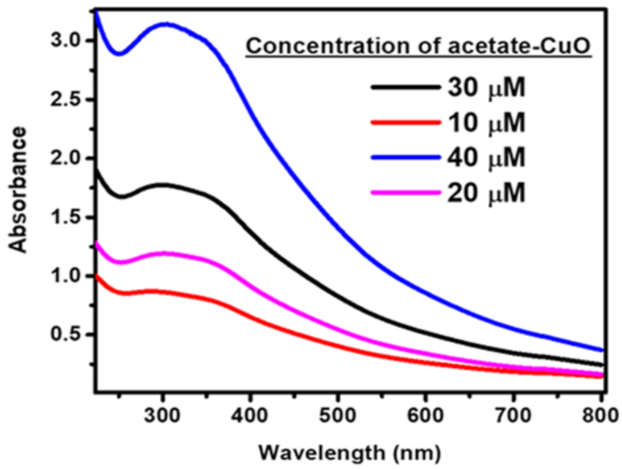

Figure 2: Absorbance spectra of synthesized acetate-CuO nanohybrid for different concentrations.

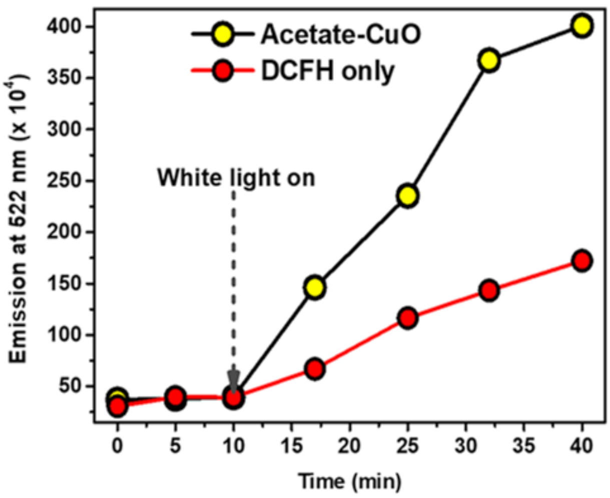

Figure 3: DCFH oxidation (monitored at 522 nm) with time in the presence and absence of acetate CuO NPs under dark (initial 10 mins) and white light (30 mins) illumination condition.

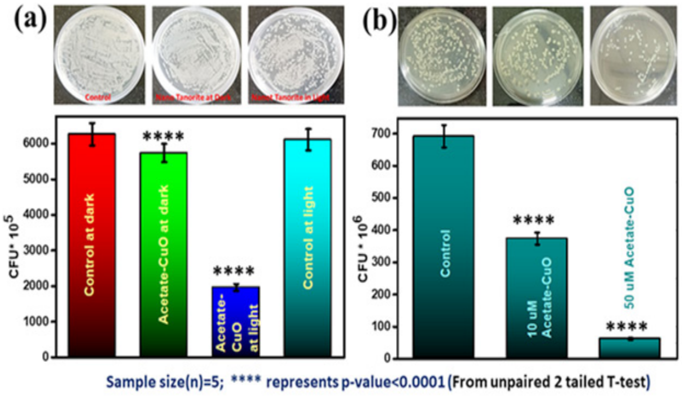

Figure 4: **(a)** Bacterial viability after treatment with acetate CuO NPs in the presence and absence of white-light irradiation (30 mins). **(b)** Dose-dependent antibacterial effect of the nanohybrid at concentrations 10uM and 50uM on MRSA under white light irradiation condition. The insets show images of MRSA plates treated with acetate CuO under different conditions.

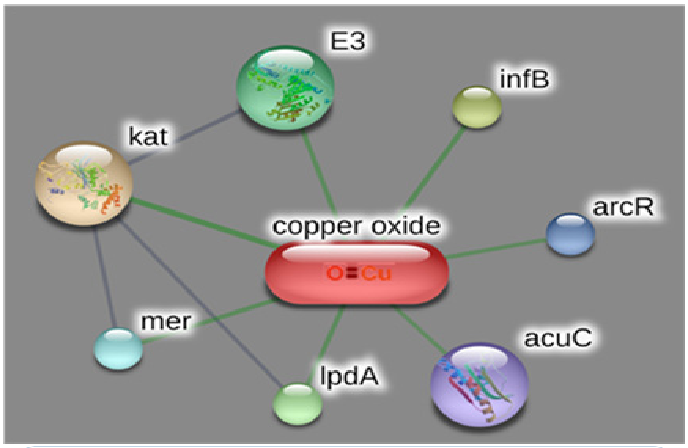

Figure 5: Compound protein interactions network of acetate-CuO nanohybrid on MRSA bacterial strain.

Table A: Effect of acetate functionalized CuO NPs on MRSA.

Protein	Activity
auk	acetoin utilization protein AcuC; Bacterial growth on acetoin and butanediol is inhibited when acuC expression is disrupted. In the absence of additional carbon sources, the bacterial fermentation product acetoin can be converted to acetate via the butanediol cycle; its decomposition is thought to be triggered by deacetylation mediated by acuC [43].
E3	dihydrolipoamide dehydrogenase; There is a strong probability that a relationship between the DLDH (E3) enzyme and its effects on virulence exists [44].
kat	catalase; Catalases are common group of enzymes that efficiently protect cells from the toxic effects of hydrogen peroxide, by decomposing it into water and oxygen to prevent cell oxidative damage [45].
mer	pyridine nucleotide-disulfide oxidoreductase
lpdA	dihydrolipoamide dehydrogenase; They have been classified according to their presence in various bacteria and eukaryotes and the properties of the enzymes are similar among the members of these domains. Additionally, it has been suggested that lpdA acts in the binding protein-dependent transport of galactose and maltose and in protecting biological membranes from oxidative degradation [46].
infB	translation initiation factor IF-2; One of the essential components for the initiation of protein synthesis. Protects formylmethionyl-tRNA from spontaneous hydrolysis and promotes its binding to the 30S ribosomal subunits. Also involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex.
arcR	Crp/FNR family transcriptional regulator; The Crp-Fnr regulators, the DNA-binding proteins, positively regulates the expression of the transcription factors [47].

Conclusion

In this study, a pure grade acetate capped CuO NP was synthesized by simple precipitation and grafting methods. XRD spectrum confirmed the formation of monoclinic crystals of CuO NPs with space group C2/C. FESEM and EDAX revealed the morphology of CuO NPs. The average SEM diameter of CuO NPs was around 38 nm that agreed fairly well with XRD and DLS data. FTIR and UV Vis spectroscopy confirmed the surface functionalization of the CuO NPs with acetate ligand. The synthesized nanohybrid was found to generate ROS under white light exposure in DCFH assay and showed excellent antimicrobial activity against MRSA bacterial strains. Consequently, acetate functionalized CuO NPs have potential for external uses as an antibacterial agent in surface coatings on various substrates to prevent microorganisms from attaching, colonizing, growing and forming biofilms for example in dwelling medical devices. This study suggests that mechanisms of antimicrobial response of acetate-CuO nanohybrid in different species of bacteria should be further explored.

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Availability of Data

All data are available on request to the corresponding author.

Conflict of Interest

The authors declare no conflict of interest.

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