Bactericidal Potency of Bioderived Carbon and Calcium Decorated on Synthesized Copper Oxide Nanocomposite from *Calotropis gigantea* against Primitive Wound Associated Pathogens

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Abstract

Commercialized antibiotics are often linked with ineffectiveness in treating chronic wound ulcer especially associated multi-drug resistant pathogens. Photocatalytic nanoparticles have become current trade attention as potent antibacterial agent. This study investigates the bactericidal potency of green synthesised CuO nanoparticles from *Calotropis gigantea* (*C. gigantea*) leaves extract at different calcination temperatures (i.e. 400°C and 500 °C) against MDR and non-MDR wound related pathogens. A well-defined oval shape CuO nanoparticles were obtained at 500 °C (CuO-500C) with present of calcium and carbon atoms were characterized using the X-ray powder diffraction, electron microscopy, and energy-dispersive X-ray spectroscopy. FTIR and UV-Vis analysis successfully confirmed phenol and carbonyl group are the stabilizing phytochemical agents. Further antimicrobial performance of CuO-500C sample was observed against non-MDR and MDR pathogens. CuO-500C sample found to markedly inhibit biofilm formation of *S. aureus*. A significant reduction of non-MDR Gram-positive bacterial colonies observe starts from 6 h exposure and bactericidal activity was noticed at 12 h exposure. Furthermore, constant prolong free radicals release was observed up to 30 days which might provide advantage against long-term antimicrobial application. Overall, it proves that high concentrated natural carbon and calcium wrapped green synthesised CuO nanocomposite demonstrated potential as a strong antimicrobial agent for combating pathogens associated with open wound infections especially in long- term basis.

1 Introduction

Open wound ulcers such as arterial ulcers, chronic ulcers, diabetic foot ulcers, diabetic infections, pressure ulcers, skin disorders, skin infections, surgical wounds, surgical infections, traumatic wounds and venous ulcers are the most common wound injuries leading to human morbidity and mortality including amputation and death [1]. Medicare cost estimates for all wound type's therapies ranged from \$28.1 to \$96.8 billion where the highest expenses were for surgical wounds followed by diabetic foot ulcers [1]. It is hardly treatable when the wound is infected by multidrug-resistant (MDR) and non-MDR skin pathogens [2, 3, 4]. Broad-spectrum antibiotics such as vancomycin, oxacillin, penicillin, cefoxitin and chloramphenicol are ineffective to control the growth of MDR skin pathogens and are not preferred in colonized open wound ulcers [5]. Besides of that, non-MDR Gram-positive *Staphylococcus aureus* (*S. aureus*) is also one of the most common skin pathogens responsible for the infectious wound ulcers [6]. Of the 137 of wound swab samples studied at University of Gondar Referral Hospital, Northwest Ethiopia, total 136 pathogens were isolated which were mainly consist of Gram-negative (57 %) and Gram-positive (43 %) [7]. Recent study showed that, roughly 23,000 deaths a year in the United States (US) and more than 33,000 in Europe recorded due to antibiotics failure in treating MDR strain infected ulcers [8].

Increasing antibiotic resistance has stimulated research addressing green synthesised copper oxide (CuO) with different morphologies as a bactericidal agent to overcome open wound ulcers (Table 1). The rise of green synthesised nanomaterials in biomedical field can be a genuine support in facilitating the healing process of open wound and ultimately repairing the injured tissue. Currently, highly moisturised antimicrobial dressings including those that contain of metals or metal oxides nanoparticles are used locally to manage skin ulcers infection [40, 41, 42, 43]. It helps in accelerating the wound recovery process by killing mechanisms of metal ions release and generation of reactive oxygen species (ROS) [14]. Other than that, the calcination temperature had a large effect on physicochemical and antimicrobial properties of metal and metal oxides nanoparticles [44]. In previous studies, F.G. El Desouky et al. (2020) determined that cerium oxide (CeO₂) crystallite size increases when the calcination temperature is increasing till 500°C [45] and Yu et al. (2003) found that higher calcination temperature improves crystallization of the anatase titanium dioxide (TiO₂) and achieve highest photocatalytic activity at 700°C [46]. It is also stated by Saravanan et al. (2015) that the high calcinations temperature results in larger average crystalline size of CuO nanoparticles [47]. Moreover, Jiao et al. (2018) identified that poorly crystallized and small grain size of CuO and CeO₂ with high specific surface area (SSA) were obtained for low calcining temperature [48].

Table 1
Physicochemical and antimicrobial properties of green synthesised copper oxide (CuO) nanoparticles

Precursor	Reducing/capping agent	Calcination temperature	Size of NPs (nm)	Shape of NPs	Antimicrobial activity	Killing mechanism	Toxicity	Application	Refs
Cupric Nitrate	<i>Calotropis gigantea</i>	400°C	20–30	Spherical	Nil	Nil	Nil	Dye-sensitized solar cells	[9]
Copper (II) acetate monohydrate	<i>Oak Fruit Hull (Jaft)</i>	500°C	Nil	Quasi-spherical	Nil	Nil	Nil	Photocatalytic	[10]
Copper nitrate trihydrate	Banana Peel	400°C	50–85	Spherical	Nil	Nil	Nil	Photocatalytic	[11]
Copper sulphate dehydrate	<i>Ocimum basilicum</i>	Nil	70	Spherical	ZOI for <i>E. coli</i> : 9.8 mm and <i>S. aureus</i> : 7.2 mm	Copper ions	Nil	Food and medical	[12]
Cupric nitrate	<i>Gloriosa superba</i> L.	400°C	5–10	Spherical	ZOI at 1000 µg/100 µL: <i>K. aerogenes</i> : 15.67 mm, <i>P. desmolyticum</i> : 5.33 mm, <i>E. coli</i> : 13.67 mm and <i>S. aureus</i> : 6 mm	Copper ions	Nil	Antibacterial	[13]
Copper (II) sulphate pentahydrate	<i>Malus Domestica</i>	Dried under lamp	18–20	Spherical	ZOI at 100 µg/ml: <i>E. coli</i> : 17 mm and <i>S. aureus</i> : 19 mm	Cu ²⁺ ion release, electrostatic interaction and ROS generation	Nil	Biological agent	[14]
Copper nitrate	<i>Drypetes sepiaria</i>	Nil	18	Nil	Nil	Nil	Nil	Photocatalytic	[15]
Copper nitrate	<i>Catharanthus Roseus</i> Leaf Extract	400°C	23	Nanorod	Nil	Nil	Nil	Catalytic material	[16]
Copper sulfate	<i>Phoenix dactylifera</i> L extract	400°C	20–28	Spherical and irregular	Nil	Nil	Nil	Nil	[17]
Copper sulfate	<i>Callistemon viminalis</i> Leaf Extract	60°C	423	Nil	At 1024 µg: ZOI for <i>E. coli</i> : 14 mm and <i>A. baumannii</i> : 12 mm	Nil	Nil	Nil	[18]
Cu (NO ₃) ₂ ·3H ₂ O	<i>Tinospora crispa</i> leaves extract	450°C	10–40	Spherical	Nil	Nil	Nil	Photocatalysts	[19]
Cu (NO ₃) ₂ ·3H ₂ O	<i>Abutilon indicum</i> leaf extract	400°C	16.78	Spherical	<i>S. aureus</i> , <i>Klebsiella</i> and <i>B. subtilis</i> with ZOI of 10 mm, 14 mm and 15 mm	Copper ions	Nil	Photo-catalytic, antimicrobial and antioxidant	[20]
Copper sulfate	<i>Lemongrass</i> Leaf extract	Nil	5.67–9.10	Spherical	Nil	Nil	Nil	Photocatalysts	[21]
CuSO ₄ ·5H ₂ O	<i>Momordica charantia</i> fruit extract	Nil	61.48	Nanorod	ZOI for <i>B. cereus</i> : 31.66 mm	Copper ions	Nil	Biomedical	[22]
Copper acetate monohydrate	<i>Psidium guajava</i> leaf extract	400°C	2–6	Spherical	Nil	Nil	Nil	Photocatalysts	[23]
Copper sulfate	<i>Adiantum lunulatum</i> whole plant extract	Nil	6.5	Spherical	Nil	Nil	Nil	Nil	[24]

Precursor	Reducing/capping agent	Calcination temperature	Size of NPs (nm)	Shape of NPs	Antimicrobial activity	Killing mechanism	Toxicity	Application	Refs
Cupric nitrate	<i>Plectranthus amboinicus</i> leaves extract	450°C	5–30	Spherical and circular	ZOI for <i>E. coli</i> : 13.65 mm, <i>K. pneumoniae</i> : 13.40 mm, <i>P. aeruginosa</i> : 13.05 mm, <i>S. aureus</i> : 13.25 mm, <i>S. phogenes</i> : 12.55 mm, and <i>B. Subtilis</i> : 12.80 mm	Cu ²⁺ , OH ⁻ radicals, O ₂ ²⁻ ions and hydrogen peroxide (H ₂ O ₂)	Nil	Drug	[25]
Hydrated copper chloride	<i>Saraca indica</i> Leaves	100°C	40–70	Spherical	Nil	Nil	Nil	Fluorescence emitting materials	[26]
Copper sulfate	<i>Cassia auriculata</i> leaf extract	Nil	23	Spherical	Nil	Nil	RAW 264.7 cell lines: low cytotoxicity up to 200 µg/mL	Drug delivery vehicle	[27]
Copper nitrate	<i>Juglans regia</i> leaf extract	300 to 500°C	80	Spherical	MIC value of 1.78% w/v	Particles attachment	Nil	Medicine and food packaging	[28]
Copper sulfate	<i>Phyllanthus Amarus</i> leaf extract	Nil	20	Large aggregated particles	ZOI for <i>B. subtilis</i> : 31 mm, <i>S. aureus</i> : 28 mm, <i>E. coli</i> : 24 mm and <i>P. aeruginosa</i> : 25 mm	Amines and carboxyl groups	Nil	Nil	[29]
Copper nitrate	<i>Aloe vera</i> leaf extract	Nil	20–30	Spherical	At 100 µg: ZOI for <i>A. hydrophila</i> : 22 mm, <i>P. Fluorescens</i> : 19 mm and <i>F. branchiophilum</i> : 17 mm	Copper ions	Nil	Antimicrobial agents	[30]
Copper chloride salt	<i>Calotropis gigantea</i> floral extract	Nil	25–35	Spherical	Nil	Nil	Zebrafish embryos: 50% viability rate for 50 mg/l	Nil	[31]
Copper sulfate	<i>Convolvulus periculus</i> L. leaves	400°C	15–30	Spherical	MIC for <i>S. aureus</i> : 1.56 and <i>E. coli</i> : 6.25 µg/ml	ROS generation	Nil	Catalysts	[32]
Copper (II) acetate	<i>Lantana camara</i> flower extract	400°C	15–40	Spherical	Nil	Nil	Nil	Catalysts	[33]
Cu (CO ₂ CH ₃) ₂ ·H ₂ O	<i>Azadirachta indica</i> leaf extract	400°C	40	Spherical	Nil	Nil	Nil	Nutritive agent	[34]
Copper nitrate trihydrate	Black tea extract	500, 600, 700 and 800°C	22–39	Spherical	Nil	Nil	Nil	Nil	[35]
Copper acetate	<i>Magnolia champaca</i> floral extract	60°C	20–40	Spherical	Nil	Nil	Zebrafish embryonic: 90% and 60% viability rate for 350 mg/l and 750 mg/l	Nil	[36]

Precursor	Reducing/capping agent	Calcination temperature	Size of NPs (nm)	Shape of NPs	Antimicrobial activity	Killing mechanism	Toxicity	Application	Refs
Cupric nitrate trihydrate	<i>Caesalpinia bonducella</i> seed extract	450°C	Nil	Rice	ZOI for <i>S. aureus</i> : 25 mm and <i>Aeromonas</i> : 34 mm	ROS generation and particles interaction	Nil	Electrochemical and biological	[37]
Copper sulphate pentahydrate	<i>Melia azedarach</i> leaf extract	80°C	14–20	Nil	ZOI for <i>E. coli</i> : 16 mm, <i>P. mirabilis</i> : 4 mm, <i>Salmonella</i> : 11 mm and <i>C. tetani</i> : 7 mm	Nil	Nil	Nil	[38]
Copper sulphate pentahydrate	<i>Morus nigra</i> leaf extract	80°C	14–20	Nil	ZOI for <i>E. coli</i> : 12 mm, <i>P. mirabilis</i> : 13 mm, <i>Salmonella</i> : 12 mm and <i>C. tetani</i> : 6 mm	Nil	Nil	Nil	[38]
Copper sulfate	<i>Cymbopogon citratus</i> (CLE) leaf extract	80°C	11.4–14.5	Spherical, hexagonal and oval	Survival rate of <i>S. aureus</i> -1 (MRSA-1): 51.4% at 1500 µg/mL, <i>S. aureus</i> -2 (MSSA-2): 32.41% at 1500 µg/mL and <i>E. coli</i> -336: 45.27% at 1000 µg/mL	Copper ions	Nil	Biomedical	[39]

Therefore, the goal of this work is to present an alternative of simple and low-cost green synthesis for the rapid one-step fabrication of highly pure and efficient CuO/C/Ca nanocomposites by aqueous extract of medicinal plant *Calotropis gigantea* (*C. gigantea*). The traditional remedies of *C. gigantea* medicinal plant in treating open wound ulcers was well documented [9]. The physicochemical properties of different calcined CuO samples were studied. Additionally, the antimicrobial properties of the CuO samples were tested against non-MDR and MDR pathogens.

2 Materials And Methods

2.1 Preparation and characterization of green synthesised CuO/C/Ca nanocomposite

Copper (II) Nitrate Trihydrate (Merck) was used as precursor for green synthesis of CuO nanocomposite. The non-MDR and MDR pathogens of *S. aureus* 29213, *E. coli* 25922, *P. aeruginosa* 27853, *K. pneumoniae* 700603 and MRSA 38591 were obtained from American Type Culture Collection (ATCC). Methylene blue (Merck) was used for photocatalytic experiment. The fresh leaves of *C. gigantea* plant were collected from Perai Pulau Pinang, Malaysia. The species was authenticated by the expert of Unit Herbarium, Pusat Pengajian Sains Kajihiyayt USM Pulau Pinang. (Herbarium No.: 11843). Green CuO nanoparticles were synthesized according to the following procedures [9, 49]. Copper (II) Nitrate Trihydrate was dissolved in filtered *C. gigantea* leaves extracts at boiling temperature of 60–80°C. The black colored powder samples which annealed at 400°C and 500°C were denoted as CuO-400C and CuO-500C, respectively. X-ray diffraction (XRD) patterns of CuO nanoparticles and powdered *C. gigantea* leaves were recorded with X-ray diffractometer (Bruker D8) by operating in reflection mode with a Cu K α radiation (40 kV, 30 mA) diffracted beam monochromator, using a step scan mode with step size of 0.030° in the range of 10° to 90°. Average crystallite sizes of CuO nanoparticles were obtained using Debye-Scherrer Equation [1]:

$$d = \frac{K\lambda}{\beta \cos \theta} \quad \text{Equation 1}$$

where K= 0.9 is the shape factor, λ is the X-ray wavelength of Cu K α radiation (0.1541 nm), θ is the Bragg diffraction angle, and β is the FWHM of the respective diffraction peak. Scanning electron microscopy (SEM Fei Quanta FEG 650) and transmission electron microscopy (TEM FEI TECHNAI F20 G2) were used for the morphology and microstructure observation of the green CuO nanoparticles, respectively. Semi-quantitative analysis of nanoparticles was carried out by energy-dispersive X-ray spectroscopy (EDAX) which is equipped with SEM machine. The primary detection of CuO nanoparticles and natural compound of leaves extract such as carbonyl and phenol group were investigated using UV–Vis spectrophotometer (Varian) at room temperature in the range of 200–700 nm. The functional groups involved in green synthesis and stabilization of CuO nanoparticles were examined using FTIR spectroscopy (Perkin Elmer). The FT-IR spectra of the green samples were recorded in the range of 4000–400 cm^{−1} by the KBr pellet method.

2.2 Minimum inhibitory concentration/minimum bactericidal concentration

The MIC/MBC of CuO nanoparticles were defined by broth dilution method on 96-well plates [50]. The MIC absorbance reading at 980 nm for CuO wavelengths was recorded before and after 24 h incubation. Besides, the MIC/MBC at concentration between 10 mg/mL and 20 mg/mL for commercial CuO and green CuO-500C samples were investigated under visible light condition. The tolerance levels against green CuO nanoparticles was determined by using the stated formula:

Tolerance = MBC/MIC

2.3 Time-kill assay

The antimicrobial performance of green CuO-500C sample against time was evaluated according to the following time-kill assay method [50]. The adjusted *S. aureus* bacterial suspension to 0.5 McFarland standard turbidity was used and diluted with CuO solution with final concentration of 20 mg/mL.

2.4 Assessment of biofilm formation

The biofilm mass of *S. aureus* bacterial strain treated with 20 mg/mL of CuO-500C sample was evaluated by crystal violet staining assay as described by NH Harun et al. (2020) [50].

2.5 Kirby-Bauer disc diffusion assay

Antibacterial activity of green CuO nanoparticles and commercial CuO (<10 µm, Sigma Aldrich) were evaluated against *S. aureus* using disk-diffusion susceptibility test according to the CLSI MO2-A11 (Clinical and Laboratory Standard Institute MO2-A11) guideline [51]. Subsequently, 2.5 mg/mL and 10 mg/mL sample's solution were prepared for antibacterial studies. Oxoid™ Cefoxitin antimicrobial susceptibility disk with concentration of 30µg was introduced as a positive control.

2.6 Determination of photocatalytic activity

The photocatalytic activity of green synthesised CuO-500C nanoparticles was studied by degradation of methylene blue (MB) aqueous solution [52]. In brief, about 100 mg of nanoparticles was immersed in 10 ppm of 40 mL of MB and kept in dark for a while to attain an equilibrium adsorption state. Photodegradation efficiency of CuO-500C nanoparticles was evaluated under normal room temperature condition between 1 hour and 1 month. The catalytic degradation of MB was observed by measuring UV-Visible spectra at regular time intervals.

3 Results And Discussion

3.1 Characterization of the green synthesised CuO/C/Ca nanocomposite

The morphology of the green CuO nanoparticles was studied using scanning electron microscopy and transmission electron microscopy (Fig. 1(e), Fig. 1(f) and Table 2). The SEM images (Figs. 1(a) and (c)) show that the CuO-400C sample have mixture of rod and quasi-spherical shaped nanoparticles and that CuO-500C sample are mainly oval in shape with agglomerated nano-sized morphology. A histogram showing the particle size of distribution for CuO-500C nanoparticles which mainly fall between 20 and 22 nm (Fig. 1(h)). Figure 1(g) shows the TEM lattice fringes with distance of 0.254 nm for CuO-500C nanoparticles. It was found that rod-shaped microstructures resulted in a slight decrease in the cidal properties against *S. aureus* which is further explained in detail on antimicrobial investigation part (figure S1). It is due to the reduced available SSA. Whereas, oval structures can increase the available SSA for improved antimicrobial reaction. In this step, energy-dispersive spectroscopy (EDAX) analysis was used to evaluate the chemical composition and the purity of the prepared samples. The EDAX profile of the green-synthesised CuO confirms the presence of Cu and O, which are about 17.52 wt.% and 17.92 wt.%, respectively, in CuO-400C and 67.23 wt.% and 25.05 wt.%, respectively, in CuO-500C (Figs. 1(b) and (d)). The EDAX profile of CuO exhibits a strong signal for Cu and O atoms. Some weak peaks for C, Ca, S, Cl, K and Mg atoms are seen for CuO green nanoparticles indicates the participation of *C. gigantea* leaves extract phytochemical compounds during the green synthesis. The CuO nanoparticles are well encapsulated by *C. gigantea* leaf extract (Fig. 1(a)), and this similar phenomenon is reported in previous research, where Au nanoparticles are well dispersed in *G. mangostana* peel extract [53].

Table 2
CuO nanoparticles size and shape

Sample	XRD crystallite size (nm)	Average TEM size (nm)	TEM shape
CuO-400C	24.1	Rod = Length: 58.46, width: 24.13; quasi-spherical: 9.73	Rod and quasi-spherical
CuO-500C	24.1	Diameter: 25.48, length: 21.25	Oval

Diffraction peaks with Miller indices of CuO-400C and CuO-500C samples are presented in Fig. 2(a). CuO-400C and CuO-500C are observed with 12 characteristic peaks at 32.32°, 35.50°, 38.71°, 45.01°, 48.37°, 53.29°, 58.15°, 61.09°, 65.56°, 67.90°, 72.16° and 75.13°, which correspond to the crystal surfaces (110), (− 111), (111), (202), (− 202), (020), (202), (− 113), (− 311), (220), (311) and (004), respectively. Green CuO particles have monoclinic crystalline structure with ICDD number of 01-089-5897. It has the following lattice parameters: a = 4.686486, b = 3.421156, c = 5.129263, α = 90°, β = 99.413°,

$\gamma = 90^\circ$, and d-spacing value of 2.52761 Å. Some additional X-ray diffraction peaks was observed in the powdered *C. gigantea* leaf sample pattern which demonstrated the CuO nanocomposite being well encapsulated and interacted with natural compounds such as carbon and calcium. These natural compounds could further accelerate antimicrobial activity of CuO nanoparticles synergistically [54, 55, 56]. The crystallite size of CuO nanoparticles was estimated from the XRD pattern using the Scherer's Equation at highest FWHM peak (Table 2).

The FT-IR analysis was performed to investigate participation of phytochemical compounds in stabilising and reducing green CuO nanoparticles (Fig. 2(b)). A sharp peak at 3438 cm^{-1} was assigned to O–H stretching polyphenols (flavonoids). The peaks between 1633 cm^{-1} and 1765 cm^{-1} were assigned to carbonyl group. The absorption peaks at 1385 cm^{-1} was assigned to vibration mode of esters. Besides absorption peaks between 1110 and 1115 cm^{-1} belongs to biomolecules of *C. gigantea* leaves extract [57, 58]. The low reported absorption peak at 540 cm^{-1} is characteristic of CuO group [59, 60].

The UV–Vis absorbance spectra of *C. gigantea* extract and green CuO sample were illustrated in Fig. 2(c). *C. gigantea* extract was referred as a control solution which does show two prominent absorbance peaks in UV spectrum at 206 nm (carbonyl compounds) and 269 nm (phenolic compounds) [61]. Solution with CuO nanocomposites illustrated a sharp distinct absorbance peak at 233 nm which belongs to natural carbon [62]. The produced nanoparticles can be identified by the appearance of small band in their respective spectra which was around 307 nm for CuO-500C nanocomposite [57].

3.2 Minimum inhibitory concentration/minimum bactericidal concentration

The wound associated pathogens are a significant global public health threat with serious wound care and management incurring huge economic costs. *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae* and MRSA are the prevailing microbial pathogens that occur in patients with open colonised wounds. Gram-positive *S. aureus* bacteria are among the most common pathogens associated with open wound infections [63]. Overall results prove that high concentrated green CuO/C/Ca nanocomposites show more enhanced biocidal activity against various wound pathogens. The preliminary MIC results of CuO-400C exhibit that CuO nanoparticles possess considerable inhibition effect on the *S. aureus* colony counts. The MIC for the CuO-400C is 5 mg/mL for *S. aureus*. At a dosage of 10 mg/mL, the result does not show any antibacterial activity (figure S1 (b)), where MBC cannot be achieved at this amount. The antimicrobial activity of CuO-500C is more pronounced than that of CuO-400C, and the MIC/MBC against *S. aureus* is 5 mg/mL/20 mg/mL (figure S1). Our MIC/MBC results have shown that the CuO-400C nanoparticles produced at low annealing temperature is less effective in killing microbes at low dosage compared with the CuO-500C sample due to reduced SSA [64]. The strong antimicrobial effect of CuO-500C sample was attributed to the smaller size and the oval shape which is produced at high calcination temperature. The surface reactivity increases due to the size and the shape of the particles. Previously, Azam et al. (2012) revealed that the small CuO particles synthesised at 400°C have a zone of inhibition radii twice that of particles produced at 700°C and that the zone of inhibition decreases with increasing annealing temperature from 400°C to 700°C [44]. N. Nasihatsheno (2019) identified that the calcination temperature affects the size and the shape of the final products and that the variation in the reaction temperature from 400°C to 600°C leads to different particle sizes and morphologies of CuO nanoparticles [65]. Sung Woo Oh et al. (2007) pinpoint in their research that the crystallite size of CuO increases until the calcination temperature reaches 500°C and that no significant change in crystallite size is observed at high calcination temperatures ($> 500^\circ\text{C}$) [66]. Furthermore, the CuO samples prepared at annealing temperatures of 300°C and 400°C exhibit similar morphological features (e.g. size and shape), but those prepared at 500°C exhibit different morphological changes [66].

So, in next set of experiment the antimicrobial performance of CuO-500C sample against non-MDR Gram-negative and MDR pathogens were discussed. The tolerance level based on MBC/MIC shows that the tested strains such as *E. coli*, *P. aeruginosa* and MRSA are not likely sensitive to bactericidal agents. Table 3 illustrates the tested non-MDR and MDR strain. It is evident from Table 3, *S. aureus* and *K. pneumoniae* is more susceptible to CuO-500C nanocomposite compare to other tested pathogens. This study recommends that killing mechanisms of CuO/C/Ca nanocomposite towards different species of pathogens should be further investigated.

Table 3
MIC and MBC of green CuO-500C sample against various pathogen associated with open wound infections

Strain	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
<i>S. aureus</i> 29213	5	20	4
<i>E. coli</i> 25922	0.625	5	8
<i>P. aeruginosa</i> 27853	0.15625	10	64
<i>K. pneumoniae</i> 700603	1.25	5	4
MRSA 38591	0.3125	2.5	8

Observation on *S. aureus* bacterial survival for green CuO-500C and commercial CuO samples at concentration of 10 mg/mL and 20 mg/mL under fluorescent visible light were captured after 24 h incubation. After 24 h of exposure (figure S2 (b)), green CuO-500C sample had a higher activity in the light rather than the dark (figure S1 (c)), except for the commercial CuO that had no effect on bacterial reduction.

From here it can be concluded that, CuO-500C which has smaller size and uniform oval shape stand as most potential antimicrobial agent than CuO-400C sample. It also has been identified that the strong antimicrobial effectiveness of green CuO-500C sample under fluorescent light illumination is most likely due to oxidative stress caused by ROS which was activated through photocatalytic reaction [60].

3.3 Time-kill assay

The time-dependent bactericidal activity of optimized green CuO-500C sample were determined against *S. aureus*. The outcomes of the experiment are shown in Figs. 3, and S3. A significant reduction of $\leq 2.5 \log_{10}$ in the bacterial colonies proportionally with time were observed for CuO green sample. A reduction in viable count from $4.3 \log_{10}$ to $2.5 \log_{10}$ is captured after 6 h and complete killing at 12 h. Results demonstrate that a high dosage of CuO-500C (i.e. 20 mg/mL) are required to eliminate 100% bacterial counts within 12 h. The untreated bacterial strains show no reduction in colony counts even after 24 h of incubation period. As captured in the time-kill analysis, a significant reduction of non-MDR Gram-positive *S. aureus* colonies were achieved at 6 h exposure.

3.4 Inhibition of biofilm formation

The bacterial biofilm formation was studied using method of crystal violet assay. CuO-500C sample exhibited significant reduction of OD when compared to the negative control sample (Fig. 4). Results showed that the CuO-500C sample was found to markedly inhibit biofilm formed by *S. aureus*. The antimicrobial action of green CuO nanoparticles might be generally falls within killing mechanisms from free metal oxide ions release and generation of reactive O species (ROS; $\cdot\text{O}^{2-}$, $\cdot\text{OH}^-$, Cu^{2+} and Ca^{2+}) [14]. Further investigation on metal ions release (Cu^{2+} and Ca^{2+}) is recommended.

3.5 Kirby-Bauer disc diffusion test

The antimicrobial activities of green CuO-500C and commercial CuO samples at two different concentration (2.5 mg/mL and 10 mg/mL) and *C. gigantea* leaves extract were examined on *S. aureus*. The ZOI reveals that CuO-500C sample was effective towards Gram-positive pathogen when increasing the concentration amount from 2.5 mg/mL to 10 mg/mL. The previous MIC/MBC results agrees with the finding in this study, in which the CuO nanoparticles antimicrobial effectiveness are concentration-dependent (Table 4 and figure S4). In accordance with Kirby-Bauer disc diffusion findings, the enhanced antimicrobial activity was seen for green CuO-500C sample at 10 mg/mL when compared with other samples. It might be attributed to rise in more free metal oxides ions and ROS at highest concentration of green nanoparticles.

Table 4

ZOI (mm) of green CuO nanoparticles against *S. aureus*

Green sample	ZOI (mm) at 2.5 mg/mL	ZOI (mm) at 10 mg/mL
Green CuO-400C	6	7
Green CuO-500C	6	7.33
Commercial CuO	6	6.17
<i>C. gigantea</i>	6.33	6.33
Negative control	6	6
Positive control	10	

3.6 Photocatalytic activity of CuO nanoparticles for degradation of methylene blue (MB)

The photodegradation efficiency of CuO-500C nanoparticles has been studied on the degradation of MB aqueous solution (Fig. 5). The color of the MB slightly decreased after 1 hour at normal room temperature condition and a sharp absorbance peak at wavelength of 665 nm was dropped significantly. Our methylene blue photocatalytic experiment demonstrated that the green synthesised CuO nanoparticles were found more active for the degradation of MB in a relatively long interval of time which is about 1 month. It might be attributed to the slow and steady release of free radicals at normal room temperature condition. The performance of 73.33 % for MB color removal was reached at 1-month duration (Table 5), which indicated that natural carbon and calcium wrapped CuO-500C nanoparticles could prolong the release time of free radicals in steady manner for long-term antimicrobial application and create stable environment that might provide biocompatible advantage [67]. It's also believed that overproduced hydroxyl radicals in short duration of time has reactive and hazardous free radicals which causes disturbance to wound-healing related cells and fibroblast [68]. Successful control on steady and slow release of free radicals such as $\cdot\text{O}^{2-}$ and $\cdot\text{OH}^-$ from this green synthesised nanoparticles might present with biocompatible properties towards human cells and can accelerate wound healing properties [69]. Besides of that, the green synthesised CuO-500C nanoparticles also might be a promising antibacterial photocatalytic agent for wound dressing application.

Table 5

The absorbency of MB during photodegradation activity

Exposure time (h)	0	1	2	3	744 (1-month)
MB absorbency at 665 nm	0.285	0.261	0.233	0.223	0.076
Degradation ratio (%)	0	8.42	18.25	21.75	73.33

4 Conclusions

This study reveals the potential of bioderived carbon and calcium decorated on synthesized copper oxide nanocomposite (CuO-500C) as antimicrobial agent against non-MDR and MDR wound associated pathogens. This bactericidal agent works effectively in concentration-dependent manner and demonstrates

strong biofilm inhibition properties against Gram-positive non-MDR strain. The control on steady and slow release of free radicals from this green synthesised nanoparticles might be beneficial biocompatible advantage that can accelerate wound healing properties. Further understanding on photodegradation efficiency of CuO-500C nanoparticles under irradiation of direct sun light or ultra-violet light is needed especially on ROS release ($\cdot\text{O}_2^-$ and $\cdot\text{OH}^-$) profile.

Abbreviations

C. gigantea: *Calotropis gigantea*, CuO: copper oxide, C: carbon, Ca: calcium, ROS: reactive oxygen species, SSA: specific surface area, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration, XRD: X-ray diffraction, MDR: multi-drug resistant, *E. coli*: *Escherichia coli*, *K. pneumoniae*: *Klebsiella pneumoniae*, *S. aureus*: *Staphylococcus aureus*, *P. aeruginosa*: *Pseudomonas aeruginosa*, MRSA: Methicillin-resistant *Staphylococcus aureus* and MB: methylene blue

Declarations

Ethics approval and consent to participate

Not applicable.

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Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to the patent application for methods of making and using of copper oxide nanocomposites formed by green synthesis but are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Authors' contributions

G Ambarasan Govindasamy carried out the green sample preparation, sample characterization and the antibacterial assays, included bacterial preparation, MIC, MBC, time kill-assay, biofilm inhibition tests and Kirby-Bauer disc diffusion method. Nor Hazliana Harun and Srimala Sreekantan assist in the procedures. Rabiatal Basria S. M. N. Mydin is the principal investigator contributes in the experimental design, writing process and gave final approval of this paper for publication. All authors have given approval to the final version of the manuscript.

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Figures

Figure 1

Morphology and green synthesis process of CuO; (a) SEM image of CuO- 400C (5 μm), (b) EDAX of CuO-400C, (c) SEM image of CuO-500C (5 μm), (d) EDAX of CuO-500C, (e) TEM images of CuO-400C (20 nm), (f) TEM images of CuO-500C (20 nm), (g) TEM lattice fringes of CuO-500C and (h) Histogram of CuO-500C particle size distribution

Figure 2

(a) XRD diffraction peaks. [: *C. gigantea* leaves, : CuO, : Additional peaks appeared after green synthesis]; (b) FTIR spectra; and (c) UV-Vis absorbance spectra

Figure 3

Time-kill curves against *S. aureus* strains using 20 mg/mL of CuO-500C sample for 0.5 h (30 min), 3 h, 6 h, 12 h and 24 h treatment periods. These data represent mean ($\pm$ SD) of three replicates.

Figure 4

Crystal violet assay to assess the biofilm inhibition activity of samples against pre-growth bacterial strains biofilm for 24 h. These data represent mean ($\pm$ SD) of three replicates.

Figure 5

UV-Visible spectra for the degradation of methylene blue (MB) by CuO-500C nanoparticles

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