Calcination Temperatures, Compositions and Antimicrobial Properties of Heterostructural ZnO–CuO Nanocomposites from Calotropis Gigantea Targeted for Skin Ulcer Pathogens

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Research

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Abstract

An eco-friendly green route is employed for the successful synthesis of heterostructured ZnO-CuO nanocomposites using Calotropis gigantea plant and the investigation of their antimicrobial properties against skin ulcer pathogens. Binary ZnO-CuO nanocomposites prepared at calcination temperature of 300 °C exhibited superior antimicrobial effect on S. aureus, whereas the negative control sample did not show any antibacterial activities. High ZnO nanoparticles of 75 wt.% ZnO-CuO nanocomposites showed zero count of Staphylococcus aureus at a minimum inhibitory concentration of 0.625 mg/mL and minimum bactericidal concentration (MBC) of 2.5 mg/mL. Interestingly, the 75 wt.% ZnO-CuO nanocomposites exhibited strong antimicrobial activity against multi-drug resistant pathogens, with MBC ranging from 0.3125 mg/mL to 1.25 mg/mL. A time-kill assay captured a reduction in viable count from 4.3 log 10 to 1.3 log 10 after 12 h of incubation for S. aureus. Elucidating the antimicrobial activities could be useful for identifying novel ways to incorporate ZnO-CuO nanocomposites in polymers for applications in biocide materials, such as for wound dressing. Further, molecular studies are needed to explain the underlying biocidal mechanism of ZnO-CuO nanocomposites especially in the presence of Cu 2+ and Zn 2+.

Introduction

Ulcerative skin infections arising from colonisation and development of gram-positive bacteria, gram-negative bacteria and multdrug-resistant bacteria are significant health care problems that seriously affect human skin. A prospective quantitative study reported on the prevalence rates of skin pressure ulcers (PUs) at 15.5% in Kuala Lumpur, Malaysia (2013) [1], 33% in Palestine (2017) [2] and 16% in Bandung, Indonesia (2017) [3]. Skin infection was found in 60 (74.0%) of the collected samples from the PUs of hospitalised patients and was mainly constituted with Enterobacteriaceae strains (49.0%) such as Escherichia coli (E. coli), Klebsiella pneumonia (K. pneumonia), Enterobacter spp. and Proteus spp.; followed by Staphylococcus aureus (S. aureus) (28.0%) and non-fermenting GNB (23.0%), mostly Pseudomonas aeruginosa (P. aeruginosa), Acinetobacter spp. and Methicillin-resistant S. aureus (MRSA) [4, 5, 6, 7]. PUs are open infected wounds that develop on the skin as result of pressure on one spot of the body for too long or from friction on the skin. Findings of new inorganic oxide antimicrobial agents synthesised from natural plant became one of the remarkable alternatives for infectious skin treatments of PUs as such options are rich in numerous varieties of metal oxides that release ions and in reactive oxygen species (ROS), such as hydroxyl radical (•OH) and superoxide (•O2−) which cause increased permeability, rupture and cell death of microorganisms [8, 9].

Recently, the incorporation of inorganic metal and metal oxides in sponges [10], hydrogels [11, 12] and bandages [13, 14] have gained priority in research because of their advantages as antimicrobial agents for treating locally infected skin ulcers. Mixed inorganic metal and metal oxides are effective disinfectants because of their relatively non-toxic profile, chemical stability and efficient antibacterial activity (Table 1). Binary antimicrobial agents (e.g. CuO and ZnO and Ag and ZnO) have been highlighted over single antimicrobial agents given the strong synergic effect of the former in eliminating bacterial
colonies at low concentration [10, 24, 38], more pronounced wound healing [10], lower cytotoxicity [10],
better biocompatibility [24] and improved cell viability which is safe for human application [24].
Therefore, this paper focuses on the preparation of green synthesised binary ZnO-CuO nanocomposites
using Calotropis gigantea (C. gigantea) leaf extract and investigates the microbial activity of these
nanocomposites upon culturing with skin ulcer pathogens such as E. coli, K. pneumonia, S. aureus, P.
aeruginosa and MRSA. Further, the effect of different compositions and calcination temperatures on ZnO-
CuO nanocomposites were explored with respect to their prospective antimicrobial application.

**Experimental Plans**

**Preparation of the leaves extract and binary inorganic oxides**

For this present investigation, the whole plant of *C. gigantea* was collected from Perai Pulau Pinang,
Malaysia and was identified by the expert of Unit Herbarium, Pusat Pengajian Sains Kajihayat USM Pulau
Pinang. (Herbarium No.: 11843). In this experiment work, 5 g of *C. gigantea* leaves were added to 100 mL
of deionized water and boiled for 60 min at temperature of 90-100°C using hot plate [39, 40]. Then, 50 mL
of filtered leaves extracts were taken and boiled to 60-80°C using a stirrer-heater. Binary inorganic oxides
ZnO-CuO nanocomposites were prepared by adding Copper (II) Nitrate Trihydrate and Zinc Nitrate
Hexahydrate into the extract solutions simultaneously and then boiled until it reduced to paste. These
pastes were calcinated in an air heated furnace for 2 h [39, 40]. In 1st stage, ZnO-CuO nanocomposites
were prepared at fixed rotation speed and composition of 50 wt.% of ZnO and 50 wt.% of CuO by varying
the calcinations temperature (300°C, 400°C and 500°C). In 2nd stage, the weight percentage of binary
oxides ZnO-CuO (25 wt. %, 50 wt. % and 75 wt. % of ZnO) was varied with constant rotation speed and
calcinations temperature of 300°C.

**XRD analysis**

The crystal phases of ZnO-CuO nanocomposites were studied using X-ray diffraction (XRD), Bruker D8
powder diffractometer operating in reflection mode with a Cu Kα 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°. The
crystallite size was estimated from the XRD pattern using the Scherer's 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 Kα radiation (0.1541 nm), θ is the Bragg
diffraction angle, and β is the FWHM of the respective diffraction peak.

**Minimum inhibitory concentration/minimum bactericidal concentration determination and tolerance level**
Antibacterial activity of ZnO-CuO nanocomposites against *S. aureus* 29213, *E. coli* 25922, *P. aeruginosa* 27853, *K. pneumonia* 700603 and MRSA 38591 were assessed using broth dilution method on 96-well plates as described by NH Harun et al. (2020) with slight modifications [41]. The bactericidal and bacteriostatic capacity of the samples was determined by the tolerance level [41].

**Time-kill assay**

The antibacterial activity of ZnO-CuO nanocomposites against time was carried out using time-kill assay as illustrated in protocol before [41]. The adjusted *S. aureus* bacterial suspension to 0.5 McFarland standard turbidity was used and diluted with samples solution with final concentration of 2.5 mg/mL.

**Results And Discussions**

**Calcination temperatures and composition of heterostructural ZnO-CuO nanocomposites**

Seven characteristic peaks of ZnO and five characteristic peaks of CuO were found in green ZnO-CuO samples prepared at different calcination temperatures (300, 400 and 500 °C) as shown in Figure 1. Diffraction peaks at 31.87°, 34.54°, 36.37°, 47.59°, 56.59°, 62.92° and 65.89° which correspond to crystal surfaces (100), (002), (101), (102), (110), (103) and (200) belonged to ZnO. Conversely, CuO was detected at 35.62°, 38.83°, 57.49°, 61.42° and 67.96°, which correspond to crystal surfaces (–111), (111), (202), (–113) and (220). The XRD pattern of ZnO-CuO nanocomposites confirms the presence of pure ZnO and CuO. Furthermore, few additional peaks were observed at 23.65°, 25.69°, 27.73°, 29.47° and 40.78° from Figures 1 and 2. This outcome is possibly due to the presence of the phytochemical element of *C. gigantea* leaves as a capping and reducing agent [39]. The additional peaks detected at 29.47° and 40.78° are attributed to the natural graphene-like carbon present in the ZnO-CuO nanocomposites [42] as carbon is a main phytochemical element in the leaves of the *C. gigantea* medicinal plant [43, 44]. Natural carbon in binary ZnO-CuO nanocomposites could further enhance the synergic effect on antimicrobial activity [45, 46]. However, these peaks lessened at higher calcination temperatures.

Prominent diffractive peaks on the differential ratio of binary ZnO and CuO nanocomposites are indexed in Figure 2. Six characteristic peaks of ZnO for sample 75ZnO25CuO-300C were identified at 31.72°, 34.45°, 36.25°, 47.35°, 56.41° and 62.71° and corresponded to crystal surfaces (100), (002), (101), (102), (110) and (103). Another two characteristic peaks of CuO at 38.62° and 67.78° corresponded to crystal surfaces (111) and (220). For sample 25ZnO75CuO-300C, 31.72°, 34.45°, 36.25°, 47.35°, 56.41°, 62.71° and 68.05° peaks, respectively belonged to the (100), (002), (101), (102), (110), (103) and (201) indices of ZnO nanoparticles. Conversely, the diffractive peaks of CuO detected at 35.68°, 38.62°, 58.33°, 61.27° and 65.80° corresponded to crystal surfaces (–111), (111), (202), (–113) and (–311). The peak intensity is drastically increased with higher amounts of ZnO or CuO in the binary ZnO-CuO nanocomposites (Figure 2), thereby indicating the variation in composition (25, 50 and 75 wt. % of ZnO) during green synthesis.

**Superior antimicrobial properties of heterostructural ZnO–CuO nanocomposites**
The antimicrobial efficacy of binary 50ZnO/50CuO nanocomposites at three different calcination temperatures (300°C, 400°C and 500°C) were initially characterised by *S. aureus* minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) as presented in Table 2. The MIC of 50ZnO/50CuO-300C and 50ZnO/50CuO-400C for *S. aureus* were 2.5 mg/mL, except for 50ZnO/50CuO-500C at 5 mg/mL. Moreover, the MBC of all green synthesised 50ZnO/50CuO samples for *S. aureus* was at 20 mg/mL. *S. aureus* colonies were counted less at a concentration of 5 mg/mL for the sample 50ZnO/50CuO-300C prepared at low calcination temperature (300°C) relative to the 50ZnO/50CuO-400C and 50ZnO/50CuO-500C samples (Figure S1). The effect of smaller sized nanoparticles generated at low calcination temperature is suggested to possibly enhance surface reactivity in killing the microbes [47, 48, 49]. Particle size is crucial in antimicrobial activity effectiveness. Azam et al. (2012) and Salah et al. (2011) verified that smaller particle sizes mean greater efficacy in inhibiting bacterial growth, a feature that is possibly associated with the larger surface areas of nanoparticles [50, 51].

Next, further characterisation of the differential ratios of binary ZnO and CuO nanocomposites at a calcination temperature of 300 °C were presented in Figure S2 and Table 2. The MIC of 25ZnO/75CuO-300C, 50ZnO/50CuO-300C and 75ZnO/25CuO-300C were 5 mg/mL, 2.5 mg/mL and 0.625 mg/mL for *S. aureus*, respectively. Similar to the MIC values, the 25ZnO/75CuO-300C and 50ZnO/50CuO-300C green samples had MBC values of 20 mg/mL and the counterpart for the 75ZnO/25CuO-300C sample was 2.5 mg/mL for *S. aureus*. The 75ZnO/25CuO-300C sample exerted a higher bactericidal effect against the *S. aureus* strain at the lowest MIC/MBC values (0.625 mg/mL/2.5 mg/mL). The antimicrobial activity was further enhanced by increasing the amount of ZnO nanoparticles in the binary compound (ZnO-CuO). The phenomenon observed can be explained by the fact that the binary 75ZnO/25CuO-300C nanocomposites are highly diffusible and generate more Zn$^{2+}$ [19]. Moreover, Cu$^{2+}$ ions bind the cell wall of host cells through surface proteins and enter the cell [19]. Subsequently, the change in the metabolism of cells leads to the microbe's cell death [19].

Further antimicrobial analysis of 75ZnO/25CuO-300C on selected skin ulcer pathogens are shown in Table 3. These pathogens are commonly associated with skin ulcer disease [4, 5, 6, 7]. The MIC values of the green synthesised ZnO-400C for *E. coli*, *P. aeruginosa*, *K. pneumonia* and MRSA were at 0.3125, 0.15625, 0.625 and 0.15625 mg/mL, respectively. By contrast, the MBC values were 2.5, 0.3125, 1.25 and 0.3125 mg/mL, respectively. Furthermore, the MIC amounts for the 75ZnO/25CuO-300C sample were 0.625, 0.15625, 0.625 and 0.15625 mg/mL for *E. coli*, *P. aeruginosa*, *K. pneumonia* and MRSA, respectively. MBC values with 2.5, 0.3125, 1.25 and 0.3125 mg/mL were also observed for this green binary inorganic oxide sample. The tolerance level according to the MBC/MIC ratio showed that all tested microbes are sensitive to bactericidal agents except for the CuO-500C sample against *E. coli*, *P. aeruginosa* and MRSA and the ZnO-400C sample towards *E. coli*. Table 3 indicates that for all tested microbes, only the tolerance levels for 75ZnO/25CuO-300C sample were less than 4, and these values identifies the sample as a strong bactericidal agent relative to other samples (ZnO-400C and CuO-500C).

Moreover, higher MBC values of the CuO-500C sample against all tested microbes possibly transpire from the slow Cu$^{2+}$ ion release from CuO nanoparticles [52]. Clearly, the ZnO-400C and 75ZnO/25CuO-300C
samples show very promising results against all tested microbes. That outcome may arise from the ZnO nanoparticle's larger surface to volume ratio and the penetration of the cell membrane of the bacteria by its ions. Furthermore, the ZnO-400C sample showed better antimicrobial activity relative to the CuO-500C counterpart at a similar concentration. Some studies reported that the antimicrobial effectiveness of green synthesised inorganic oxide nanoparticles depends on particle dosage, size and treatment condition, such as calcination temperatures. This situation could be the one of the reasons for the higher antimicrobial activities of ZnO and ZnO-CuO over that of CuO particles.

Additionally, the antimicrobial activities of the ZnO-400C and 75ZnO/25CuO-300C samples are much better than that of the CuO-500C sample alone towards multi-drug resistant strains P. aeruginosa, K. pneumonia and MRSA. That outcome is obviously due to the high diffusion of Zn$^{2+}$ ions in the medium. The poor activity of CuO particles within a shorter duration suggested that the time requirement for water diffusion and subsequent Cu$^{2+}$ release influence efficacy. The attacking delay was also associated with the cell walls of gram-negative strains. Studies have reported that gram-negative bacterial strains exhibit higher resistance or tolerance against nanomaterials compared with gram-positive bacteria [53] because of the lipopolysaccharide situated in the outer membrane of the former [54]. The cytoplasmic membrane, which is inherent to gram-negative bacteria, significantly maintains cellular viability. Hence, gram-negative microbes are not readily attacked by free radicals or Cu$^{2+}$. More time and concentrated Cu$^{2+}$ ions are thus required to effectively decompose the cell membrane of the bacteria. The antimicrobial activity of ZnO, CuO and ZnO-CuO nanoparticles is due to the electrostatic interaction between positively charged zinc and copper ions (Zn$^{2+}$ and Cu$^{2+}$) and negatively charged microbial cell membranes [21]. In addition, the antimicrobial activity of inorganic oxide nanoparticles relies on the generation of ROS as well [17, 19].

In the time-kill assay results were presented in terms of the changes in the log$_{10}$ CFU/mL of viable S. aureus colonies and indicated that the green synthesised binary 75ZnO/25CuO-300C sample exhibited significant bactericidal activity. The outcomes of the time-kill assay were captured in Figure S3. Figure 3 presents the time-kill curve graph for the strain. A reduction in viable count from 4.3 log$_{10}$ to 3.4 log$_{10}$ was captured after 6 h of incubation for S. aureus. By 12 h, only 1.3 log$_{10}$ of bacterial colonies were seen. At 24 h, the bacteria were completely killed. Therefore, the effective control of gram-positive S. aureus bacteria was achieved by the synergistic combination of 75 wt.% of ZnO and 25 wt.% of CuO nanoparticles with the presence of phytochemical constituents such as cardiac glycosides, tannins, saponins, terpenes, flavonoids and phenolics in the leaf extract of the C. gigantea medicinal plant [55, 56, 57, 58].

**Conclusions**

In summary, heterostructural ZnO-CuO nanocomposites prepared at a calcination temperature of 300 °C and with a composition of 75 wt.% of ZnO and 25 wt.% of CuO demonstrated significant antimicrobial property against skin ulcer pathogens. The mechanisms that underlie the biocidal activity of ZnO-CuO nanocomposites were reflected by the presence of Cu$^{2+}$ and Zn$^{2+}$ ions and ROS. This finding could
reduce the environmental bio-burden in a hospital atmosphere, specifically in relation to pressure ulcerative skin infections.

**Abbreviation**

*C. gigantea*: *Calotropis gigantea*, *E. coli*: *Escherichia coli*, *K. pneumonia*: *Klebsiella pneumonia*, *S. aureus*: *Staphylococcus aureus*, *P. aeruginosa*: *Pseudomonas aeruginosa* and MRSA: Methicillin-resistant *S. aureus*.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**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 and compositions of binary nanocomposites formed by green synthesis but are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare no conflict of interest.

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**Authors’ contributions**

G Ambarasan Govindasamy carried out the green sample preparation, sample characterization and the antibacterial assays, included bacterial preparation, MIC, MBC and time kill-assay. Nor Hazliana Harun and Srimala Sreekantan assist in the experimental procedures. Rabiatul Basria S. M. N. Mydin 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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Tables
| Mixed oxides       | Route of synthesis                  | Size (nm) | Shape                        | Calcination temperature | Killing mechanism | Antimicrobial activity                                      | Refs  |
|--------------------|-------------------------------------|-----------|------------------------------|--------------------------|-------------------|-------------------------------------------------------------|-------|
| ZnO/CuO            | Green route-Theobroma cacao seed bark extract | 20-50     | Spherical and rice grains    | 400°C                    | Nil               | Nil                                                         | [15]  |
| CuO-ZnO            | Biological route-Cnici benedicti     | 28        | Spherical                    | Nil                      | Nil               | S. aureus, E. coli, P. aeruginosa sa and C. albicans        | [16]  |
| Cu-doped ZnO       | Solution combustion-Clerodendrum infortuntum extract | 17.49     | Rod                          | 200°C                    | Generatio n of reactive oxygen species | Klebsiella, B. subtilis and T. harzianum                  | [17]  |
| Cu-doped ZnO       | Solution combustion-Clerodendrum inerme | 20.73     | Rod                          | 200°C                    | Generatio n of reactive oxygen species | E. coli, S. aureus, Klebsiella, B. subtilis, A. niger and T. harzianum | [17]  |
| ZnO/CuO            | Green route-Mentha longifolia leaf extract | At 10 wt.% CuO: 10, ZnO: 14 | Spherical                   | 60°C                     | Nil               | S. aureus and E. coli                                      | [18]  |
| CuO-ZnO            | Sol-gel                             | 15.99     | Uniform particle             | 500°C                    | Production of Zn$^{2+}$ ions and reactive oxygen species | S. aureus and E. coli                                    | [19]  |
| Copper-Doped ZnO   | Deposits                             | 50 and 100, 100 and 600 | Globular structure consisting | Nil                      | Oxidative stress caused by ROS, Zn$^{2+}$, Cu$^0$, | E. coli                                        | [20]  |
| Material | Preparation Method | ZnO: CuO (Cu<sup>1+</sup>, Cu<sup>2+</sup>) Formed | Cu<sup>2+</sup> and Zn<sup>2+</sup> Ions Released | Temperature | Bacterial Targets |
|----------|---------------------|---------------------------------|---------------------------------|-------|-----------------|
| Zeolite\ZnO-CuO | Facile method | ZnO: 25.9, CuO: 56.2 | CuO and ZnO formed on surface of zeolite cubic structure | 450°C | B. subtilis [21] |
| Mesoporous silica SBA/CuZnO | Impregnation | 2 µm | 2D hexagonal and honeycomb structure | 550°C | E. coli and S. aureus [22] |
| CS/Zn-Cu | Physico-chemical | 1.7-23.7 | Nil | 60°C | B. cinerea [23] |
| ZnO/Ag | Green route-<i>Mirabilis jalapa</i> leaf extract | 19.3-67.4 | Plates, sheets, and spherical | Nil | K. pneumonia and S. aureus [8] |
| Zinc oxide/silver | Oxalate decomposition | ZnO: 40.07 ± 9.70, Ag: 37.46 ± 12.02 | Spherical | ZnO: 500°C, Ag: 40°C | Ag<sup>+</sup> ions release and ZnO produces ROS | S. aureus and E. coli [24] |
| Ag/ZnO | Depositio-n-precipitation | Length: 100-400 and width: 50-200 | Rod-like structures | 60°C | Nil | E. coli, S. aureus, P. aeruginosa, DREC and MRSA [10] |
| Ag-ZnO | Green route-<i>Azadirachta indica</i> gum | 15, pore diameter: 70-500 | Spherical, porous and honeycomb structure | 500°C | Nil | Nil [25] |
| Ag/ZnO | Stepwise microwa | ZnO: 1 µm; Ag: hollow | ZnO: hollow | 40°C | Silver ion and E. coli and | [26] |
| Method | Process | Size | Shape | Description | Temperature | Antibacterial Activity | Reference |
|--------|---------|------|-------|-------------|-------------|------------------------|------------|
| Ag-ZnO Bent | Microwave-assisted synthesis | Ag: 9-30 and ZnO: 15-70 | Aggregated particle | 70°C | Nil | E. coli and E. faecalis | [27] |
| Honeycomb doped silver and zinc | Wet ceramic powder process in combination with co-firing | Nil | Honeycomb structure with a porous surface | Nil | Nil | E. coli | [28] |
| Ag-Cu | Green route-flower aqueous extract of A. haussknechtii | 24.82 ± 4.85 | Berries like | Nil | Electrostatic interaction and production of reactive oxygen species | E. coli, S. aureus and P. aeruginosa | [9] |
| Ag-Cu | Nanocasting | Core diameter: 25, Cu shell: 3.7 | Rough pores | 80°C | Silver ions generate ROS and copper induces hydroxyl radicals | E. coli and B. subtilis | [29] |
| Ag/Cu | Chemical reduction & impregnation | 1-30 & 100-200 | Spherical | 200°C | Penetration of Ag NPs, Ag⁺ and Cu²⁺ ions release | C. albicans, E. coli and S. aureus | [30] |
| Ag-Cu/TNTs | Microwave assisted alkaline hydrothermal process & UV photodeposition | TNTs: 7.5-10 thickness and ~5 inner diameter | Bundle | 80°C | Reactive oxygen species & superoxide radical anion | S. aureus | [31] |
| Material | Preparation Method | Shape | Size | Temperature | Electrostatic Interaction and Production of Reactive Oxygen Species | References |
|----------|--------------------|-------|------|-------------|---------------------------------------------------------------|-------------|
| Cu-Ag   | Green route-flower aqueous extract of A. haussknechtii | Needle | 33.79 ± 18.73 | Nil | Electrostatic interaction and production of reactive oxygen species | E. coli, S. aureus and P. aeruginosa [9] |
| Ag-TiO₂ | Green route-flower aqueous extract of A. haussknechtii | Spherical | 36.99 ± 12.03 | Nil | Electrostatic interaction and production of reactive oxygen species | E. coli, S. aureus and P. aeruginosa [9] |
| TiO₂-Ag | Green route-flower aqueous extract of A. haussknechtii | Cubic | 35.55 ± 9.88 | Nil | Electrostatic interaction and production of reactive oxygen species | E. coli, S. aureus and P. aeruginosa [9] |
| TiO₂/ZnO-4A zeolite | Hydrothermal method & ion exchange process | Equiaxed | 10-50 | 500°C | Production of ROS; Zn²⁺ release and particle's penetration | S. aureus, P. fluorescens, L. monocytogenes and E. coli [32] |
| ZnO/TiO₂ | Precipitation method & sol-gel | No defined shape | 100 | 500°C | Zn²⁺ ions release | S. aureus, E. coli, K. pneumoniae, P. aeruginosa, S. paratyphi A and C. albicans [33] |
| Au-CuO | Biological synthesis using using Cnici benedicti | Spherical | 13 | Nil | Nil | S. aureus, E. coli, P. aeruginosa and C. albicans [16] |
| Graphene-ZnO | Green route- | Spherical | 25 | 100°C | Ion release & S. aureus and E. | [34] |
Crocus sativus petal extract

| Method       | Cu/Pd     | Ag/Fe       | Zinc oxide/gentamicin-CS |
|--------------|-----------|-------------|--------------------------|
| Facile method | 3         | 5-40        | 15                       |
| Hexagonal    | Nil       | Irregular-truncated triangular polyhedral nanodisks and spherical | Polyhedral |
| Metal ions release | | 50°C        | 80°C         |
| E. coli, P. aeruginosa, E. faecalis and S. aureus | | Electrostatic interaction of ions | Nil |
| [35]         |           | [36]        | [37]                     |

**Table 2**: MIC and MBC of ZnO-CuO nanocomposites against S. aureus

| Samples                  | MIC (mg/mL) | MBC (mg/mL) | MBC/MIC |
|--------------------------|-------------|-------------|---------|
| 50ZnO/50CuO-300C         | 2.5         | 20          | 8       |
| 50ZnO/50CuO-400C         | 2.5         | 20          | 8       |
| 50ZnO/50CuO-500C         | 5           | 20          | 4       |
| 75ZnO/25CuO-300C         | 0.625       | 2.5         | 4       |
| 25ZnO/75CuO-300C         | 5           | 20          | 4       |
### Table 3: MIC and MBC of binary and single inorganic oxides against different microbes

| Strain        | Samples            | MIC mg/mL | MBC mg/mL | MBC/MIC |
|---------------|--------------------|-----------|-----------|---------|
|               |                    |           |           |         |
| **S. aureus** |                    |           |           |         |
| 29213         | CuO-500C           | 5         | 20        | 4       |
|               | ZnO-400C           | 0.625     | 1.25      | 2       |
|               | 75ZnO/25CuO-300C   | 0.625     | 2.5       | 4       |
| **E. coli**   |                    |           |           |         |
| 25922         | CuO-500C           | 0.625     | 5         | 8       |
|               | ZnO-400C           | 0.3125    | 2.5       | 8       |
|               | 75ZnO/25CuO-300C   | 0.625     | 2.5       | 4       |
| **P. aeruginosa** |                |           |           |         |
| 27853         | CuO-500C           | 0.15625   | 10        | 64      |
|               | ZnO-400C           | 0.15625   | 0.3125    | 2       |
|               | 75ZnO/25CuO-300C   | 0.15625   | 0.3125    | 2       |
| **K. pneumonia** |                |           |           |         |
| 700603        | CuO-500C           | 1.25      | 5         | 4       |
|               | ZnO-400C           | 0.625     | 1.25      | 2       |
|               | 75ZnO/25CuO-300C   | 0.625     | 1.25      | 2       |
| **MRSA 38591** |                |           |           |         |
|               | CuO-500C           | 0.3125    | 2.5       | 8       |
|               | ZnO-400C           | 0.15625   | 0.3125    | 2       |
|               | 75ZnO/25CuO-300C   | 0.15625   | 0.3125    | 2       |

### Figures
Figure 1

XRD diffraction peaks of ZnO-CuO nanocomposites prepared at different calcination temperature. (a) 50ZnO/50CuO-300C, (b) 50ZnO/50CuO-400C and (c) 50ZnO/50CuO-500C
Figure 2

XRD diffraction peaks of ZnO-CuO nanocomposites prepared at different composition. (a) 25ZnO/75CuO-300C, (b) 50ZnO/50CuO-300C and (c) 75ZnO/25CuO-300C
Figure 3

Time-kill curves against S. aureus strains using 2.5 mg/mL of 75ZnO/25CuO-300C sample for 0.5 h (30 min), 3 h, 6 h, 12 h and 24 h treatment periods. These data represent mean (± SD) of three replicates.

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