

Article

Comparative Analysis of Cu₂O:ZnO Nanocomposites Synthesized via Green and Sol-Gel Methods for Antibiofilm and Antibacterial Activities

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Abstract: The increasing prevalence of antibiotic-resistant bacteria, especially in biofilm-related infections, highlights the urgent need for innovative antimicrobial materials with effective mechanisms. This study presents a comparative analysis of Cu₂O-ZnO nanocomposites synthesized via two distinct methods: a green synthesis approach employing extract of *Curcuma longa* (turmeric) rhizomes G-Cu₂O:ZnO and a sol-gel method SG-Cu₂O:ZnO. The nanocomposites were evaluated their antibiofilm and antibacterial properties. The relevance of the structural, optical, and morphological studies of the obtained nanocomposites was determined using XRD, FTIR, UV-Vis spectroscopy, and FESEM-EDX analysis. The nanocomposite sample synthesized using the green method exhibited better phase purity, uniform spherical morphology, and a smaller crystallite size range compared to those synthesized by the sol-gel method. The major distinctions are structural, where green synthesis gives a simpler binary phase structure and a better oxidation state control. The nanocomposites characterized efficient bacteria killing against both Gram-positive and Gram-negative bacteria as well as effective antibacterial activity against multi-drug-resistant bacterial species. The Uv-Vis optical properties, characterized by narrow bandgaps and localized surface plasmon resonance (SPR), enhance their antibacterial capabilities. Thus, functional groups identified by FTIR spectroscopy also support their antimicrobial property.

Keywords: Antibacterial, Antibiofilm, Green, Nanocomposite, Sol-gel

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1. Introduction

The emergence of diseases caused by antibiotic-resistant bacteria and the complications due to biofilm-associated infections are compelling people to look for new alternative means of combating bacterial infections[1]. In this regard, metal oxide nanoparticles especially Cu₂O and ZnO have received enormous attention because of their embedded physicochemical features and excellent antimicrobial properties[2]. Also, the nanocomposite shows better functionality than its individual counterparts; therefore, its potential use in biomedical applications[3,4]. Nanocomposites and various products of biomedical fields using nanocomposites are considered to be one of the most rapidly growing and innovative areas in medicinal sciences. As can be seen, they are used in the making of drug/protein-releasing coatings on implants, tissue engineering scaffolds, and in a variety of diagnostic techniques[5]. The application of nanocomposites to biomaterials could make the diagnosis and treatment processes more effective and precise, and extend the service life of such biomaterials. It can be thus expected that inorganic/organic

biodegradable nanocomposite systems will be a key element of the present society[6]. Based on the available studies, scholars are expected to uncover a set of new products in the following years which would be designed with biodegradable nanocomposites. As a fast-growing field biomedical nanocomposites are successfully enhancing many works at present time. In this case, the drug is administered to the patients through tablets, capsules, and time-release formulations[7]. The reason why such synthesis methods have been selected is substantive. The green synthesis approach also incorporates the increasing focus on safer and greener ways of making nanomaterials that require fewer hazardous chemicals and potentially even better biocompatibility. On the other hand, the sol-gel method is a very precise technique of preparing the nanoparticles and tailoring the structure of nanoparticles can easily be varied to get the best physiochemical properties for a specific application[8]. Kandav et al. (2024) synthesized metallic nanoparticles employing the green synthesis method using plant extracts, microorganisms, and biopolymers. This method is green, economical, reproducible, and versatile enabling applications in catalysis, medicines, and energy-related technologies[9]. Oleiwi et al. (2022) conducted a comparative study using Sol-gel and green synthesis techniques to prepare TiO_2 Nanoparticles. The TiO_2 nanoparticles were analyzed for crystallite size, functional groups, optical properties, and morphology [10]. Kohili et al. (2025) Green synthesis of carbon quantum dots (CQDs) from natural raw materials offers a sustainable and biocompatible approach for diverse applications[11]. The antimicrobial activity involves the bacterial strain isolation, identification, evaluation of minimum inhibitory concentration (MIC), sub-MIC, biofilm, antibiofilm, and antibacterial activities using the agar well diffusion method, providing a reliable method for analyzing the properties of the nanocomposite[12], [13], [14], [15], [16]. The present work aims to investigate the fabrication of $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite through green synthesis and sol-gel approaches. In the green synthesis, the metal nanoparticles are reduced and stabilized by *Curcuma longa* (turmeric) rhizomes, an excellent environment-friendly molecule; while sol-gel derivatization provides better control over the nanoparticle size and composition. This study demonstrates how $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposites can be used in biomedical applications to combat microbial resistance and biofilm formations further in the development of nanomaterial-based on metal oxides.

2. Materials and Methods

Copper nitrate trihydrate $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ functioned as the precursor material during the synthesis of $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite. where the minimum assay of Cu is 99 % with maximum limits of impurities of $(\text{Cl}^-) \leq 0.003\%$, $(\text{SO}_4^{2-}) \leq 0.01\%$, $(\text{Fe}) \leq 0.01\%$, $(\text{Pb}) \leq 0.05\%$, and (hydrogen sulfide) $\leq 0.1\%$. Zinc nitrate hexahydrate $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ were the minimum assay of (Zn) is 98 % with maximum limits of impurities of $(\text{HNO}_3) \leq 0.05\%$, $(\text{SO}_4^{2-}) \leq 0.005\%$, $(\text{Fe}) \leq 0.001\%$, $(\text{Cl}^-) \leq 0.005\%$, $(\text{Pb}) \leq 0.002\%$. Both materials are from Thomas Baker, India. *Curcuma longa* extract was obtained from turmeric rhizomes.

Synthesize of $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite using green method

Preparation of Plant Extract: *Curcuma longa* extract was obtained from turmeric rhizomes and evaluated in the dried form and ground into fine powder. From the powder, 1 gram was weighed and in a clean beaker, 2 grams of the powder was dissolved in 100 mL distilled water. The mixture was heated to 50°C for 1 hour with continuous stirring at 700 rpm to facilitate the extraction of *Curcuma longa*. A heating process was followed by cool-down to room temperature then vacuum filtration with filter paper to separate residues. The filtrate, which contains *Curcuma longa* extract, was used in the synthesis approach as a natural reducing and stabilizing agent.

Green Synthesis: Under vigorous stirring of 700 rpm at 25°C for one hour, one molar of copper nitrate trihydrate and zinc nitrate hexahydrate solutions were dissolved in separate beakers containing 50 ml of distilled water. Combining the two solutions to create a homogeneous mixture of copper and zinc nitrates. Slowly add the *Curcuma longa* extract to the combined solution while continuously stirring, allowing *Curcuma longa* extract to act as a reducing and stabilizing agent for both copper and zinc ions. by adding NaOH drop by drop to raise solution pH into an alkaline range of ~ 12 , facilitating the

precipitation of metal oxides. Stir the reaction mixture for 1h, maintaining the temperature at around 70-80°C. A color change into the green solution indicates the formation of the G-Cu₂O: ZnO nanocomposite. The precipitate was washed, dried at 100°C for 4 hours, and calcined at 500°C for 2 hours.

Synthesize of Cu₂O:ZnO nanocomposite using sol-gel method

The copper nitrate and zinc nitrate were dissolved in deionized water, forming a clear solution with a light blue color. Then sodium hydroxide was added dropwise while stirring the solution continuously, and the pH was maintained at ~12 to facilitate the formation of metal hydroxides with a cloudy blue color. The reaction mixture was heated to 80°C and stirred continuously, allowing the metal ions to undergo hydrolysis and condensation reactions, which resulted in the formation of a gel. Stirring continued until the gel became stable, indicating the complete transition from solution to gel with pale blue to bluish-gray color. The formed gel was then dried at 100°C for 4 hours to remove any excess water and volatiles, leading to the formation of a dry, solid precursor. The dried gel was subsequently calcined at 500°C for 2 hours in a muffle furnace. This thermal treatment enabled the decomposition of metal hydroxides into their respective oxides, Cu₂O and ZnO, while promoting the crystallization of the SG-Cu₂O: ZnO nanocomposite material.

Isolation and Identification of Bacterial Strains

Four types of bacteria strains were isolated and identified which are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*, clinical specimens are collected from appropriate sources such as pus, wound swabs, sputum, urine, and blood. For *S. aureus*, samples are inoculated onto Mannitol Salt Agar (MSA), which selects for *Staphylococcus* and differentiates *S. aureus* by mannitol fermentation, producing yellow colonies after incubation at 37°C for 24 hours. Identification is confirmed using microscopy, catalase, coagulase tests, and further biochemical tests like DNase production and mannitol fermentation. For *P. aeruginosa*, samples are cultured on *Pseudomonas* Isolation Agar and incubated at 37°C for 24 hours. Identification involves microscopy, Gram staining, catalase, oxidase, and fluorescence tests, with pyocyanin production (a blue-green pigment) as a key characteristic. *K. pneumoniae* is cultured on MacConkey Agar, incubated at 37°C for 24 hours, and identified by Gram staining, lactose fermentation, and biochemical tests such as urease, indole, and hydrogen sulfide production. *E. faecalis* samples are inoculated onto *Enterococcal* Agar, sometimes supplemented with bile salts, and incubated at 37°C for 24 hours. Identification is performed using Gram staining, catalase, bile esculin tests, and growth in high salt concentrations.

Minimum Inhibitory Concentration (MIC) and sub-MIC

The determination of minimum inhibitory concentration (MIC) and sub-MIC for bacterial isolates is determined for *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and *E. faecalis*, perform double serial dilutions using the following procedure. Prepare two antibiotics in double serial dilutions, ranging from 1 to 1024 µg/mL, by diluting a stock solution (10 mg/mL) in Mueller-Hinton broth on a microtiter plate. Inoculate each well with 20 µL of bacterial suspension, ensuring the suspension matches McFarland standard no. 0.5 (approximately 1.5×10^8 CFU/mL). For negative control wells, do not add any bacterial suspension. Incubate the microtiter plates at 37°C for 18-20 hours. After incubation, add 20 µL of resazurin dye to each well, which will indicate bacterial viability. Incubate the plates for an additional 2 hours at 37°C. After this second incubation, observe the wells for color changes. A shift from blue to pink indicates bacterial growth, while wells that remain blue suggest inhibition, indicating the MIC.

Biofilm and Antibiofilm Activity

The biofilm and antibiofilm activities of both nanocomposites were evaluated against four bacterial strains: *S.aureus*, *P.aeruginosa*, *K.pneumoniae*, and *E.faecalis*. Bacterial cultures were prepared by inoculating each strain in LB broth, followed by incubation at 37 °C for 16-18 hours. Both nanocomposites were dissolved in PBS or DMSO at concentrations ranging from 10 to 100 µg/mL and sterilized by filtration. For the biofilm formation assay, 100 µL of each bacterial suspension (1×10^6 CFU/mL) was added to a 96-well microtiter

plate and incubated at 37 °C for 24 hours to allow biofilm formation. After the incubation process, planktonic cells were carefully removed, and the wells were washed with PBS to eliminate the remaining floating cells. In the antibiofilm assay, 100 µL of either G-Cu₂O:ZnO nanocomposite or SG-Cu₂O:ZnO nanocomposite solution was added to wells containing pre-formed biofilms, and the plate was incubated for an additional 24 hours at 37°C. Control wells included untreated bacteria (positive control) and nanocomposite solutions without bacteria (negative control). After treatment, the biofilm was quantified using crystal violet staining. Wells were washed, stained with 0.1% violet crystal for 15 minutes, washed again, and the stained biofilm was dissolved using ethanol or acetic acid. Absorbance was measured at 570 nm using a microplate reader to quantify biofilm formation. To determine the minimum biofilm inhibitory concentration (MBIC₅₀), serial dilutions of the nanocomposites were tested to find the lowest concentration that inhibited at least 50% of biofilm formation. Finally, data were analyzed to compare the antibiofilm activity of G-Cu₂O:ZnO nanocomposite and SG-Cu₂O:ZnO nanocomposite for each bacterial strain, determining which nanocomposite and concentration exhibited the greatest biofilm inhibition.

Antibacterial Activity

The agar well diffusion method was utilized to assess the antibacterial activity of G-Cu₂O:ZnO nanocomposite and SG-Cu₂O:ZnO nanocomposite against *S.aureus*, *P.aeruginosa*, *K.pneumoniae*, and *E. faecalis* at the MIC concentration. Each bacterial isolate was grown in nutrient broth at 37 °C for (18-24) hours. Following this, 0.1 mL of each bacterial suspension was spread on nutrient agar plates and incubated for 24 hours. A single colony was transferred to 5 mL of normal saline to achieve a turbidity of approximately 1.5×10^8 CFU/mL. Utilizing a sterile cotton swab, the bacterial suspension was evenly spread on Mueller-Hinton agar plates and left to dry for 10 minutes. Wells (5 mm in diameter) were created in the agar, and 50 µL of nanocomposites were added to each well, with distilled water as a control. The plates were incubated at 37 °C for 18 hours. After incubation, the diameters of the inhibition zones were measured, and analyze the antibacterial efficacy of the nanocomposites.

Characterization Techniques

Techniques including different analytical methods were used to analyze the Cu₂O-ZnO nanocomposites that were synthesized. Research employed X-ray Diffraction (XRD) through a Bruker D8 Advance diffractometer equipped with Cu K α radiation ($\lambda = 1.54060$ Å). The machine worked under 45 kV and 35 mA voltage. The characterization of crystalline sample phases occurred by collecting data from 5° to 85° 2 θ at a step size of 0.015° and a scan rate of 1.5°/min. The formation of metal–oxygen bonds together with functional group detection was investigated using an FT-IR spectroscopy system operated from the Thermo Scientific Nicolet iS5 spectrometer. The spectra measured from 4000–400 cm⁻¹ using ATR (Attenuated Total Reflectance) enabled measurement through the solid sample without KBr pellets. The identification of the Cu-O and Zn-O stretching vibrations through spectrometry analysis were used to confirmed the successful synthesis of the nanocomposites. A PerkinElmer Lambda 1050 UV-Vis-NIR spectrophotometer produced the optical characteristics readings of the nanocomposites. The absorption spectra measurement took place from 250–850 nm. A quartz cuvette held the distilled water solution containing the nanocomposites which underwent sonication until they formed a uniform colloid before measurement. An optical band gap determination was made possible by drawing a Tauc plot with $(\alpha h\nu)^2$ against photon energy ($h\nu$) while utilizing the absorption coefficient value computed from the absorbance measurements. A JEOL JSM-7610F microscope under 15 kV conditions performed Field Emission Scanning Electron Microscopy (FESEM) tests to study the surface characteristics along with particle dimensions and structural elements at multiple magnification levels. The FESEM system incorporated EDX mode to analyze elemental composition and its 25 kV operation allowed detection of copper (Cu) zinc (Zn) and oxygen (O) signatures. The atomic analysis of Cu to Zn affirmed the proper nanocomposite formation and stoichiometric ratio of the Cu₂O-ZnO material.

3. Results and Discussion

X-ray diffraction (XRD) serves as the method to analyze the crystal structures in the synthesized products. The interplanar lattice spacing d can be determined through application of Bragg's law formula Equation 1 [17].

$$n\lambda = 2d \sin \theta \dots \dots \dots 1$$

The X-ray diffraction process depends on four measurements including peak order n , incident wavelength λ , interplanar spacing d , and incidence angle θ . Knowing the interplanar spacing depends on the foundation between n and λ to determine d for various crystal structures. Equation 2 of Debye-Scherrer provided the calculations for average crystallite size. The method allows scientists to determine the dimensions of crystal fragment specimens.

$$D = (k\lambda)/(\beta \cos \theta) \dots \dots \dots 2$$

The X-ray crystallite size D expressed in nanometers can be calculated through the equation using the form factor K (0.94), wavelength λ (0.154060 nm), Bragg diffraction angle θ (Radian) and the Full Width at Half Maximum (FWHM) β (Radian) of the selected peak [18]. The X-ray diffraction (XRD) patterns of G-Cu₂O:ZnO nanocomposite and SG-Cu₂O:ZnO nanocomposite are shown in Fig. 1. The comparison of these nanocomposites provides insights into the structural characteristics, phase composition, and crystallite sizes of the resulting nanocomposites. The XRD patterns of G-Cu₂O:ZnO nanocomposite revealed Cu₂O cubic crystalline structure described in the entry crystallography open database (COD) [96-101-0942] along with hexagonal crystalline structure of ZnO based on entry cod [96-230-0116]. Three prominent peaks detected at 35.027°, 36.51° and 57.45° reveal crystallite dimensions of 24.32 nm from the (002) plane and 22.94 nm from (111) together with 23.13 nm from (110) [19].

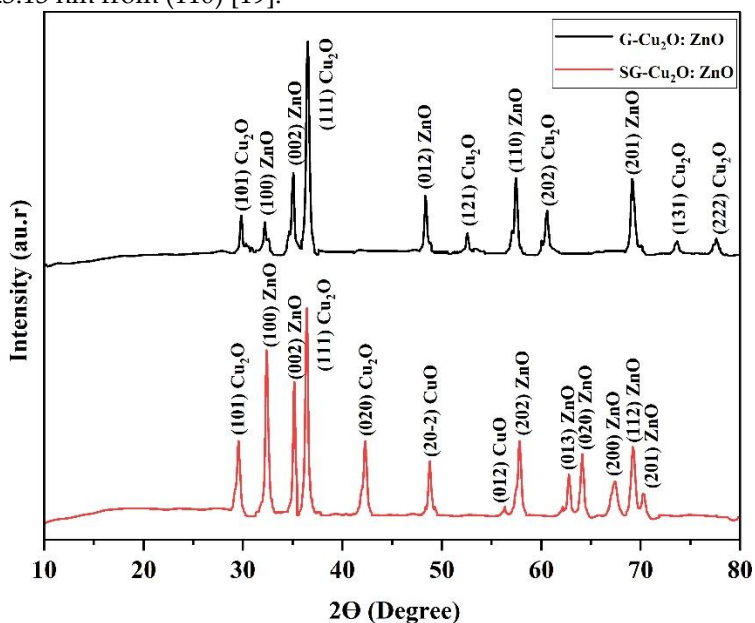


Figure 1. XRD pattern of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites.

The XRD patterns of SG-Cu₂O:ZnO nanocomposite identified a cubic and monoclinic crystal structure of Cu₂O and CuO according to the entry cod [96-900-5770] and [96-410-5683], respectively. Also, a hexagonal crystal structure of ZnO according to the entry cod [96-230-0117]. The three strongest peaks (2θ); 32.39°, 35.16°, and 36.41° correspond to planes and crystallite sizes of (100) plane: 25.55nm, (002) plane: 28.69 nm, and (111) plane: 25.95 nm, respectively[20]. Both methods produce nanocomposites containing Cu₂O and ZnO phases. The sol-gel method additionally shows monoclinic CuO, indicating partial oxidation of Cu₂O, while the green synthesis method favors Cu₂O formation without CuO. The sol-gel method yields slightly larger crystallites (26.73 nm) compared to green

synthesis (23.46 nm), suggesting more extended crystal growth. Differences in peak orientations indicate variations in crystal growth, with green synthesis showing the strongest peak at 36.51° (111) for Cu_2O , and sol-gel at 32.39° (100). Both methods yield hexagonal ZnO , with slight structural differences. The sol-gel method produces a more complex $\text{CuO}/\text{Cu}_2\text{O}$ system, while green synthesis favors a binary Cu_2O nanocomposite. The sol-gel method offers mixed copper oxide phases for tunable properties, while green synthesis provides better control over phase purity and oxidation state. The FESEM images in Fig. 2 (a-b) show the surface morphology of the synthesized samples G- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite (SG- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite, respectively). The G- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite reveals several distinct morphological characteristics when compared to the SG- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite. The particles in the SG- $\text{Cu}_2\text{O}:\text{ZnO}$ sample have a larger size and more angular structure compared to that of G- $\text{Cu}_2\text{O}:\text{ZnO}$ particles which are more uniform and nearly spherical. This irregularity causes a highly non-uniform particle morphology with clearly distinguishable changes in the particle shape and size. Additionally, the SG- $\text{Cu}_2\text{O}:\text{ZnO}$ particles gave a higher average particle size of 63.23 nm and a broader size distribution that suggests a wider variation in the diameters of particles. In terms of surface texture, the SG- $\text{Cu}_2\text{O}:\text{ZnO}$ particles appear to be somewhat more irregular, perhaps due to the sol-gel synthesis method, which can impose further surface imperfections and asymmetries. The packing density of the nanoparticles in this sample is also notably higher, with fewer void spaces between particles, suggesting a more compact arrangement that might lead to a reduced overall surface area compared to the SG- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite. The green synthesis of G- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite provides improved spherical morphology with a narrow particle size distribution of ZnO with an average particle size of 55.42 nm size. Its surface pH texture and more porous packing arrangement could be advantageous for applications that benefit from higher surface area and accessibility. Conversely, the sol-gel synthesized nanocomposite sample, displays a broader particle size distribution, rougher surface texture, and denser packing. These differences, likely due to the sol-gel process, may introduce structural variations and defects, impacting its catalytic activity, adsorption capacity, and overall functional properties. These morphological differences have demonstrated the dependency of the synthesis methods on the applicational performance of the nanocomposites. (Figure 2)

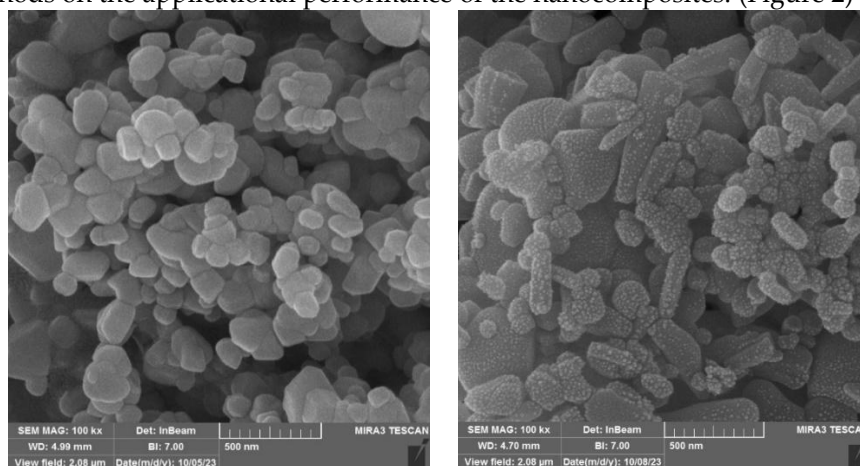


Figure 2. FESEM images of (a-left) G- $\text{Cu}_2\text{O}:\text{ZnO}$ and (b-right) SG- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposites.

Fig. 3 a and b show the energy-dispersive X-Ray (EDX) spectrum of G- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite and SG- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite, respectively. Also give a quantitative result corresponding to copper, zinc, and oxygen elements in a weight percentage (W%) and an atomic weight percentage (A%) of the synthesized samples. The EDX spectrum of G- $\text{Cu}_2\text{O}:\text{ZnO}$ nanocomposite sample confirms several elemental composition; Oxygen ($\text{O}_{K\alpha 1}$) at 0.540 KeV with 31.17 W%; 59.86 A%, Copper ($\text{Cu } K\alpha 1$): at 8.1 KeV with 40.20 W%; 19.44 A% and Zinc ($\text{Zn } K\alpha 1$) at 8.680 KeV with 25.17 W%; 11.83 A%, and Carbon (C): 3.47

W%; 8.87 A%. The presence of Cu, Zn, and O confirms the formation of the Cu₂O: ZnO nanocomposite structure synthesis by the sol-gel method. In the SG-Cu₂O:ZnO sample, EDX analysis shows Oxygen (O k_{α1}) with 25.98 W%, 40.91 A%; Copper (Cu k_{α1}) with 33.46 W%, 19.30 A%; Zinc (Zn k_{α1}) with 35.01 W%, 28.14 A% and Carbon (C k_{α1}) at with 5.55 W%, 11.64 A% [21,22]. In summary, both the SEM and EDX analyses validate the successful synthesis of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites. Overall, the EDX analysis confirms the successful formation of the Cu₂O:ZnO nanocomposite with some areas for potential optimization in terms of purity and particle dispersion.

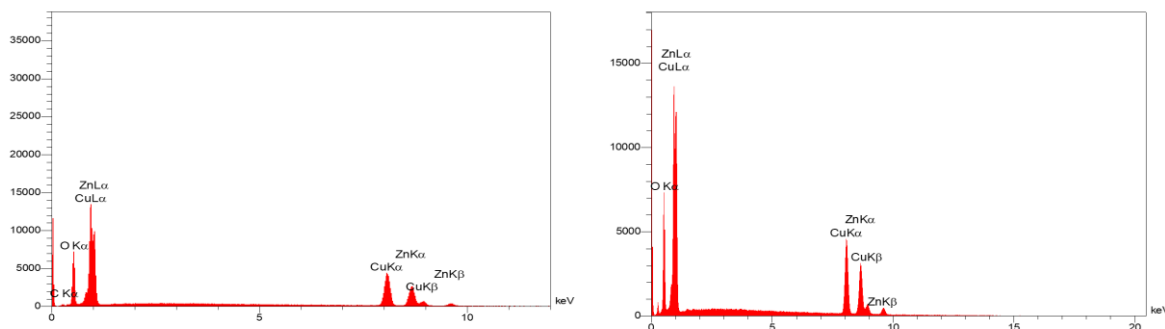


Figure 3. EDX spectrum of (a-left) G-Cu₂O:ZnO and (b-right) SG-Cu₂O:ZnO nanocomposites.

The optical properties of the nanocomposite samples were investigated using a UV-Vis spectrophotometer. The absorption spectrum with the wavelength range (190-1100 nm) of the electromagnetic spectrum and energy bandgap for the direct electronic transitions of the G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites shown in Fig. 4 a and b, respectively. They are revealing similar optical and electronic properties with significant implications for biological applications. Both nanocomposites demonstrate nanocrystalline characteristics, with G-Cu₂O:ZnO displaying an absorption peak at 521 nm and SG-Cu₂O:ZnO at 522 nm, falling in the green region of the visible spectrum (495-570 nm). The direct allowed energy bandgap (E_g) is accessed using Tauc's equation 3 by plotting $(\alpha h\nu)^2$ vs $h\nu$ via extrapolating a straight line on the energy axis [23].

$$\alpha h\nu = B (h\nu - E_g)^r \dots \dots \dots 3$$

Here: E_g is the energy gap between direct transitions, B is a constant dependent on the material type, $h\nu$ is photon energy, equal to $1240 \text{ eV} / \lambda$, and the power coefficient is symbolized by r ; The computation is based on potential electronic transitions, where a factor of $(1/2)$ represents direct allowed transitions, while $(3/2)$ represents a direct forbidden transition. The energy bandgap values of 2.12 eV for G-Cu₂O: ZnO and 2.10 eV for SG-Cu₂O:ZnO indicate strong visible light absorption, efficient electron-hole pair generation, and enhanced photocatalytic activity, all of which are advantageous for antibacterial and antibiofilm functions. In terms of antibacterial activity, the narrow bandgaps facilitate efficient visible light absorption, driving electron-hole pair generation and producing reactive oxygen species (ROS) crucial for bacterial inhibition. The localized surface plasmon resonance (SPR) at 521-522 nm contributes to enhanced interaction with bacterial membranes, localized heating, and improved cell penetration. For antibiofilm applications, the low bandgaps and strong visible light absorption allow continuous ROS generation under light exposure, disrupting biofilm formation, degrading existing biofilms, and preventing bacterial adhesion through surface modification. The optical properties also influence the minimum inhibitory concentration (MIC) values, as the narrow bandgaps reduce the concentrations needed for effective bacterial inhibition, support faster antimicrobial action, and maintain continuous ROS production. This continuous ROS generation ensures consistent antibacterial efficacy under visible light, effectively enhancing bacterial growth inhibition. Mechanistically, absorption at 521-522 nm activates photocatalytic processes, with bandgaps of 2.12/2.10 eV allowing efficient electron-hole separation and sustained ROS generation. SPR effects improve bacterial

membrane penetration, allowing photogenerated species to cause cellular damage, while photocatalytic activity further prevents bacterial adhesion and biofilm formation through surface interactions and ROS-based degradation. The practical advantages include visible light activation without UV requirements, making the materials safe for biological use, and comparable bandgaps and SPR peaks indicating similar performance across both nanocomposites. Their strong visible light absorption and antimicrobial capabilities render them versatile for medical applications, functioning effectively in varied lighting conditions and compatible with current treatment protocols. The slight differences in bandgap (2.12 vs. 2.10 eV) and absorption peak positions (521 vs. 522 nm) suggest that both synthesis methods produce materials of similar effectiveness, with the green synthesis (G-Cu₂O:ZnO) offering an environmentally friendly alternative to sol-gel synthesis (SG-Cu₂O:ZnO) while retaining comparable optical and antimicrobial properties. Together, these nanocomposites emerge as promising candidates for antibacterial, antibiofilm, and other photochemical applications in medical settings.

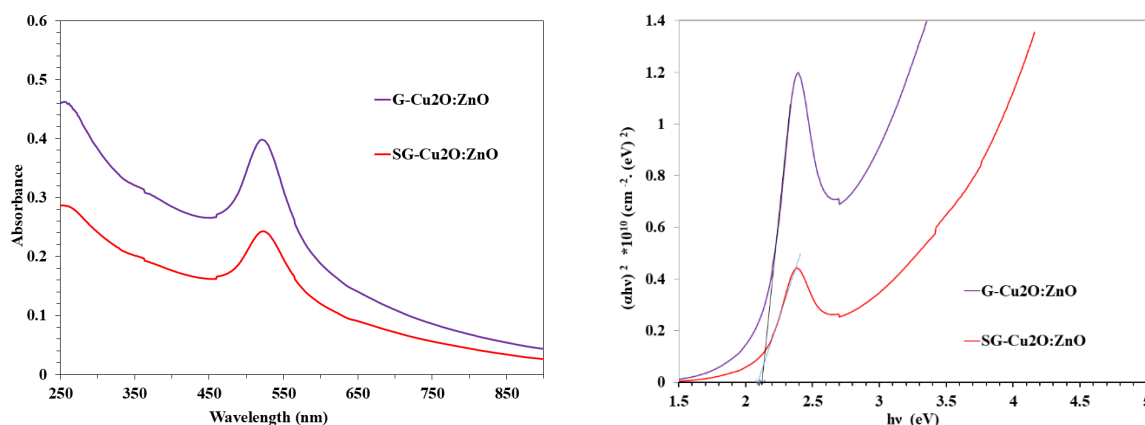


Figure 4. Optical properties (a-left) Uv-Vis absorption spectrum and (b-right) Energy bandgap (E_g) of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites.

Fourier Transform Infrared Spectroscopy (FTIR) was used to investigate the functional groups and chemical bonds of the nanocomposite samples. The FTIR spectra shown in Fig. 5 reveal significant functional groups that directly correlate with their biological activities of the G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites. The characteristic of the absorption spectra bands in the range of 400-4000 cm⁻¹, with both samples exhibiting similar patterns but with slight shifts in peak positions, indicating successful synthesis through different methods. The prominent O-H stretching vibrations observed at 3443 cm⁻¹ (G-Cu₂O:ZnO) and 3441 cm⁻¹ (SG-Cu₂O:ZnO) are crucial for biological applications as these hydroxyl groups facilitate the generation of reactive oxygen species (ROS), which play a vital role in antimicrobial activity and biofilm disruption[24]. The presence of C-H stretching vibrations at 2934/2931 cm⁻¹ and bending modes at 1388/1383 cm⁻¹ suggests organic moieties that may contribute to the material's biocompatibility and interaction with bacterial cell membranes. Particularly significant for antimicrobial applications are the metal-oxygen vibrations observed at lower wavenumbers (532/488 cm⁻¹), which correspond to Cu-O and Zn-O bonds [25]. These metal-oxygen bonds are essential for the controlled release of Cu²⁺ and Zn²⁺ ions, which are fundamental to the materials' antibacterial mechanism through bacterial cell membrane disruption and metabolic interference. The bands at 1074/1068 cm⁻¹, attributed to C-O stretching, may enhance the materials' interaction with bacterial biofilms. These functional groups collectively contribute to the material's effectiveness in minimum inhibitory concentration (MIC) studies by facilitating multiple antibacterial mechanisms, including ROS generation, ion release, and membrane interaction. For biofilm and antibiofilm activities, the presence of surface hydroxyl groups and metal-oxygen bonds is particularly important as they enable penetration into biofilm matrices and prevent bacterial adhesion. The slight variations in peak positions between the two samples

suggest different coordination environments that might influence their biological efficacy, particularly in terms of ion release rates and ROS generation capacity. This comprehensive functional group analysis supports the materials' potential as effective antimicrobial agents through multiple simultaneous mechanisms of action, making them promising candidates for various biological applications requiring bacterial growth inhibition and biofilm control.

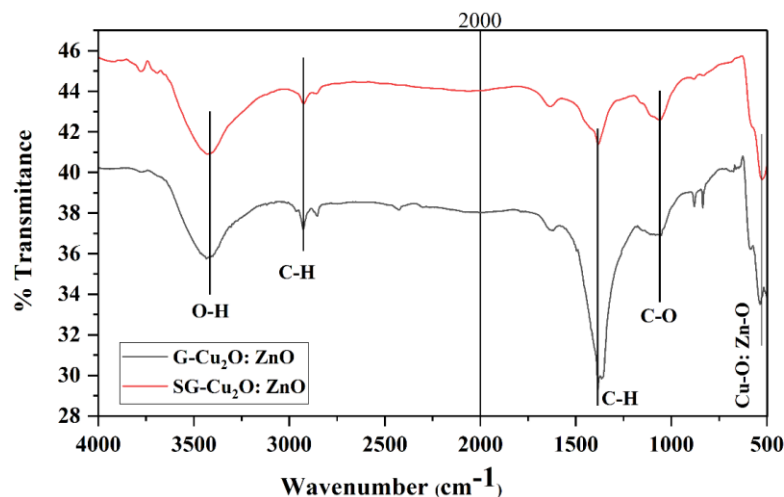


Figure 5. FTIR spectra of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites.
Isolation Efficiency and Bacterial Identification Outcomes

Isolation and identification of bacterial strains are conducted as follows: *S. aureus* is typically isolated from wound infections, skin abscesses, or nasal swabs. It appears as Gram-positive cocci in clusters on Gram staining and forms yellow, beta-hemolytic colonies on blood agar. Confirmation involves positive catalase, coagulase, DNase tests, and mannitol fermentation on MSA. *P. aeruginosa* is isolated from respiratory, burn, or urinary tract infections. It shows Gram-negative rods on staining, produces a greenish-blue pigment (pyocyanin) on nutrient agar, and has a grape-like odor. It is oxidase-positive, motile, and grows on *Pseudomonas* Isolation Agar. *K. pneumoniae* is obtained from sputum, urine, or wound samples. It shows Gram-negative rods and forms large, mucoid, lactose-fermenting (pink) colonies on MacConkey agar. It is non-motile, urease-positive, and produces hydrogen sulfide. *E. faecalis*, often isolated from UTIs, blood, or wound infections, appears as Gram-positive cocci in pairs or chains. It grows in 6.5% NaCl, turns bile-esculin agar black, and is frequently resistant to multiple antibiotics.

MIC and sub-MIC Evaluation and Antibacterial Activity

Table 1 presents the MIC and sub-MIC evaluations of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites. For *S. aureus*, the MIC for SG-Cu₂O:ZnO nanocomposite was 32 µg/mL, with a sub-MIC of 16 µg/mL, while G-Cu₂O:ZnO nanocomposite demonstrated lower values of 8 µg/mL (MIC) and 4 µg/mL (sub-MIC). This indicates that G-Cu₂O:ZnO nanocomposite is more effective at inhibiting growth. Similarly, *P. aeruginosa* showed MIC values of 32 µg/mL and 16 µg/mL for SG-Cu₂O:ZnO nanocomposite, whereas G-Cu₂O:ZnO nanocomposite had values of 16 µg/mL (MIC) and 8 µg/mL (sub-MIC), indicating higher antibacterial activity of G-Cu₂O:ZnO nanocomposite. In the case of *K. pneumoniae*, SG-Cu₂O:ZnO nanocomposite had MIC and sub-MIC values of 32 µg/mL and 16 µg/mL respectively; nevertheless, G-Cu₂O:ZnO exhibited much better efficacy, with MIC and sub-MIC values of 16 µg/mL and 8 µg/mL respectively. For *E. faecalis*, both nanocomposite samples exhibited equal MIC and sub-MIC of 8 µg/mL and 4 µg/mL against this bacterium so there is no significant difference in the efficacy of both the nanocomposites. Overall, G-Cu₂O:ZnO nanocomposite is more effective than SG-Cu₂O:ZnO nanocomposite against Gram positive *S. aureus*, Gram negative *P. aeruginosa*, and *K. pneumoniae* bacterium as suggested by the lower MIC. However, for *E. faecalis*, both

materials exhibit similar antibacterial effects. The enhanced efficacy of G-Cu₂O:ZnO nanocomposite may be attributed to its composition and structural properties, leading to more efficient bacterial inhibition.

Table 1. MIC and sub-MIC of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites.

The bacterial isolates	G-Cu ₂ O:ZnO nanocomposite (µg/ml)		SG-Cu ₂ O:ZnO nanocomposite (µg/ml)	
	MIC	Sub-MIC	MIC	Sub-MIC
<i>S. aureus</i>	8	32	16	4
<i>P. aeruginosa</i>	16	32	16	8
<i>K. pneumoniae</i>	16	32	16	8
<i>E. faecalis</i>	8	8	4	4

Evaluation of Biofilm and Antibiofilm Activity

The analysis of biofilm and antibiofilm activity of the G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites present in Table 2 showed that the ability of bacterial strains to form biofilm and react to the inhibition processes has different levels among all the tested strains. The initial OD values (before treatment) were obtained to be ranging between 0.444 ± 0.040 to 0.792 ± 0.081 which shows the variation of capability of bacterial species to form biofilms. After treatment, both nanocomposites demonstrated a remarkable reduction in biofilm formation, with G-Cu₂O:ZnO nanocomposite demonstrating higher efficiency in combating all the bacterial strains with no exception when compared to SG-Cu₂O:ZnO nanocomposite. The G-Cu₂O:ZnO nanocomposite exhibited the highest efficiency in biofilm inhibition, achieving reduction rates of 77.98% for *S. aureus* (OD reduction from 0.595 to 0.131 ± 0.044), 75.13% for *P. aeruginosa* (from 0.792 to 0.197 ± 0.004), 73.77% for *K. pneumoniae* (from 0.694 to 0.182 ± 0.003), and 78.15% for *E. faecalis* (from 0.444 to 0.097 ± 0.004). In comparison, SG-Cu₂O:ZnO nanocomposite showed moderate but significant reduction rates of 51.60%, 45.58%, 36.89%, and 26.80% for the respective bacterial strains, with final OD values of 0.288 ± 0.137 , 0.431 ± 0.100 , 0.438 ± 0.067 , and 0.325 ± 0.108 .

Table 2. Biofilm and Antibiofilm Activity of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites.

The bacterial isolates	Before treatment OD Mean $\pm$ SD	After treatment OD Mean $\pm$ SD	
		G-Cu ₂ O:ZnO nanocomposite	SG-Cu ₂ O:ZnO nanocomposite
<i>S. aureus</i>	0.595 ± 0.048	0.131 ± 0.044	0.288 ± 0.137
<i>P. aeruginosa</i>	0.792 ± 0.081	0.197 ± 0.004	0.431 ± 0.100
<i>K. pneumoniae</i>	0.694 ± 0.146	0.182 ± 0.003	0.438 ± 0.067
<i>E. faecalis</i>	0.444 ± 0.040	0.097 ± 0.004	0.325 ± 0.108

The enhanced performance of G-Cu₂O:ZnO nanocomposite can be attributed to several factors, including better dispersion, smaller particle size, and increased surface area for bacterial interaction, leading to more efficient release of active ions. All treated samples were classified as non-adherent based on the OD_c calculations, indicating that both treatments effectively prevent biofilm formation. This suggests that the nanocomposites successfully interfere with bacterial adhesion mechanisms and may disrupt the initial attachment phase of biofilm formation. The variation in effectiveness among bacterial strains might be due to differences in bacterial cell wall structure and biofilm composition, with *E. faecalis* and *S. aureus* showing higher sensitivity to treatment compared to *P. aeruginosa* and *K. pneumoniae*. The mechanism of action likely involves multiple pathways, including the release of Cu²⁺ and Zn²⁺ ions, generation of reactive

oxygen species (ROS), disruption of bacterial cell membranes, and interference with bacterial communication systems. The non-adherent classification across all bacterial strains emphasizes the potential of these nanocomposites in preventing biofilm-associated infections. However, further studies focusing on long-term stability, in vivo efficacy, and potential cytotoxicity would be valuable for developing practical applications. The consistent performance of both nanocomposites, particularly G-Cu₂O:ZnO nanocomposite, in preventing bacterial adhesion suggests promising applications in medical devices, implants, and other surfaces where biofilm prevention is crucial. This study provides strong evidence for the potential use of these nanocomposites as effective antibiofilm agents in various biomedical applications, with G-Cu₂O:ZnO nanocomposite showing particularly promising results for future development and implementation.

Evaluation of Antibacterial Activity

The antibacterial activity of Cu₂O:ZnO nanocomposites reveals several significant findings. Based on the data presented in Table 3 outlines the diameter of inhibition zones (in millimeters) for various bacterial isolates when exposed to nanoparticles synthesized using two different methods: Sol-gel and green synthesis. Additionally, the antibacterial performance of these nanoparticles is compared to azithromycin (Azm) at a concentration of 15 mg/ml. Notably, the G-Cu₂O:ZnO nanocomposite produced through the green synthesis method consistently resulted in larger inhibition zones across all bacterial isolates compared to those synthesized via the sol-gel method. *E. faecalis* exhibited the largest inhibition zones for both preparations, measuring 30 mm for the SG-Cu₂O:ZnO nanocomposite and 33 mm for the G-Cu₂O:ZnO nanocomposite. In contrast, *P. aeruginosa* showed complete resistance to azithromycin (with no inhibition zone, marked as 0 R), yet was significantly inhibited by both nanocomposites. *K. pneumoniae* demonstrated the smallest inhibition zones when exposed to both nanocomposites among all tested bacterial isolates.

Table 3. Antibacterial activity of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites.

The bacterial isolates	The diameter of the inhibition zone (mm)		
	G-Cu ₂ O:ZnO nanocomposite (15 mg/ml)	SG-Cu ₂ O:ZnO nanocomposite (15 mg/ml)	Azithromycin (15 mg/ml)
<i>S. aureus</i>	20	25	18
<i>P. aeruginosa</i>	25	28	0 R
<i>K. pneumoniae</i>	15	20	10
<i>E. faecalis</i>	30	33	20

The G-Cu₂O:ZnO nanocomposite appears more effective in generating antibacterial activity compared to the SG-Cu₂O:ZnO nanocomposite as shown in Fig. 6, likely due to variations in particle size, shape, or surface properties. Furthermore, both nanocomposites exhibited broad-spectrum antibacterial activity against Gram-positive (e.g., *S. aureus*, *E. faecalis*) and Gram-negative bacteria (e.g., *P. aeruginosa*, *K. pneumoniae*), suggesting their potential utility in combating antibiotic-resistant strains. The varying susceptibility of different bacterial species to the nanoparticles was also noted, with *E. faecalis* being the most susceptible and *K. pneumoniae* the least affected. The potential mechanisms underlying the antibacterial activity of both nanocomposites may include the generation of reactive oxygen species, disruption of bacterial cell membranes, and interference with bacterial metabolism. It is important to recognize that all tests were conducted at a single concentration (15 mg/ml), and further studies with varying concentrations would be valuable to determine minimum inhibitory concentrations and elucidate dose-dependent effects. Future research should explore the exact mechanisms of action, investigate potential synergistic effects with conventional antibiotics, and conduct in vivo studies to further evaluate the efficacy and safety of these nanoparticles as antimicrobial agents,

particularly those synthesized using the green method, showing promising broad-spectrum antibacterial activity. These findings suggest potential applications in the development of new antimicrobial treatments, especially against antibiotic-resistant strains.(Figure 6)

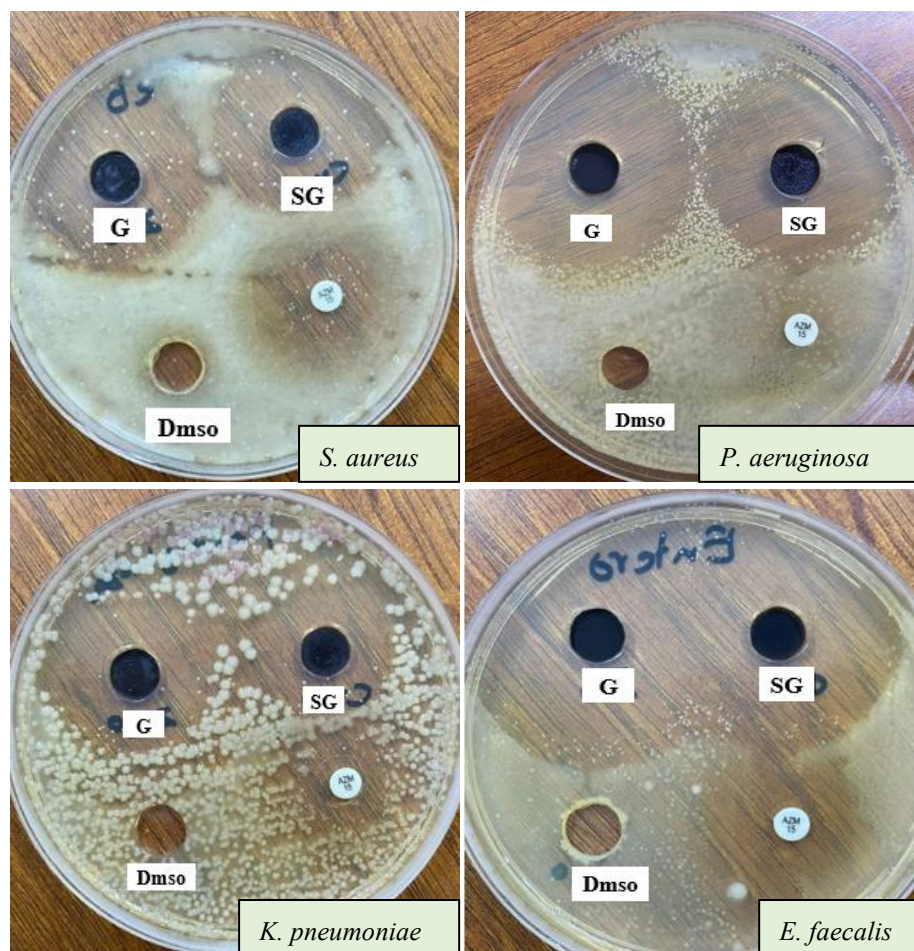


Figure 6. Antibacterial activity of G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites.

4. Conclusion

The Cu₂O:ZnO nanocomposites were successfully synthesized using green and sol-gel methods with remarkable potential as innovative antibacterial and antibiofilm agents. The XRD analysis revealed that G-Cu₂O:ZnO exhibited a binary phase system with cubic Cu₂O and hexagonal ZnO structures, while SG-Cu₂O:ZnO showed an additional monoclinic CuO phase. FESEM images demonstrated that G-Cu₂O:ZnO possessed a more uniform, spherical morphology in the nanoparticle size range, compared to SG-Cu₂O:ZnO which shows a larger nanoparticle size range with more irregular particles. UV-Vis spectroscopy revealed comparable optical properties for G-Cu₂O:ZnO and SG-Cu₂O:ZnO nanocomposites, characterized by narrow absorption bands and localized surface plasmon resonance (SPR) within the visible region. The presence of nanocrystalline structure resulted in a red shift in the SPR peaks of both samples. Moreover, results revealed that the E_g reduced as a result of the excitonic effect and narrowing of the energy bandgap. The FTIR spectrum revealed functional groups: O-H stretching (3443/3441 cm⁻¹) indicates ROS generation, while C-H vibrations (2934/2931 cm⁻¹, 1388/1383 cm⁻¹) support biocompatibility. Metal-oxygen vibration (532/488 cm⁻¹) indicates Cu-O and Zn-O bonds vital for ion release. C-O stretching. The antibacterial activity of G-Cu₂O:ZnO nanocomposite against four human pathogenic bacterial strains. Demonstrated superior antibacterial activity with lower minimum inhibitory concentrations (MICs) ranging from

8-16 µg/mL, compared to 8-32 µg/mL for SG-Cu₂O:ZnO. Notably, G-Cu₂O:ZnO nanocomposite exhibited enhanced antibiofilm activity with reduction rates of 73.77-78.15% across all tested strains, significantly higher than the 26.80-51.60% reduction achieved by SG-Cu₂O:ZnO nanocomposite. The results indicate that the green synthesis approach not only offers an environmentally friendly alternative but also produces nanocomposites with superior antimicrobial properties. These findings suggest potential applications in developing sustainable, efficient antimicrobial materials for medical applications, particularly in combating antibiotic-resistant bacterial infections and biofilm formation.

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