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CuO Doped Wollastonite Clusters for Some Anti-microbial and Anti-fungi Applications

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ABSTRACT

Wollastonite clusters doped with/without CuO (0.0-10.0 g/100 g nominal $CaSiO_3$) were prepared via precipitation route. This method is suitable for the development of wollastonite clusters, as ingenious compared with other costly methods. Wollastonite was developed after heat-treatment at 550°C/2 h, as rounded to sub rounded nano-crystals at the size of 20-50 nanometers. The cluster powders were quantitatively tested on Gram negative bacteria (Pseudomonas aeruginosa and Escherichia coli), Gram positive bacteria (Bacillus subtilis and Staphylococcus aureus) and Fungi (Aspergillus niger and Fusarium solani) using the agar diffusion technique. CuO-containing samples showed good activity against E. coli, P. aeruginosa and A. niger fungi. Gram negative bacteria Pseudomonas aeruginosa and fungi Aspergillus niger were detected to be most susceptible to the 5.0% CuO concentration with an inhibition zone diameter (IZD) between 30 mm and 22 mm, respectively. Moreover, E. coli showed an IZD of 20 mm with the 3.0% CuO. Stability test of sample 3.0% along 8 days, showed moderate resistance to B. subtilis, E. coli, A.

niger and P. aeruginosa and weak stability against S. aureus and F. solani. Clusters of wollastonite with/without CuO prepared via wet precipitation route can be considered a novel antimicrobial agent for bone implantation.

Keywords: Clusters, Wollastonite, CuO, Antibacterial, Antifungal and inhibition zone diameter

INTRODUCTION

Wollastonite (WS) is a calcium silicate-based ceramic, well-known as monocalcium silicate [WS, $CaSiO_3$]. WS has been recommended as a material that activates bone tissue regeneration owing to its excellent bioactivity, high osteo-conductivity and degradability compared to stoichiometric hydroxyapatite [HA] [1,2]. Recently, the merging of metallic antibacterial agents such as (Cu^{2+}, Ag+, Ce^{4+} and Zn^{2+}) into bioceramic materials was employed [3]. This may provide reducing cytotoxicity and microbial growth at the implant site [4]. Copper endorses high antibacterial ability by sustaining a low cytotoxicity [5]. In small quantities copper is important for various activities in the living organisms. Conversely, at higher content it affords better antibacterial effect, with enlarged cytotoxicity [6]. Among the class of silicate ceramics, wollastonite has been mostly considered owing to its noticeable properties: (i) Rapid formation of an appetite layer on the surface in comparison with other silicate ceramics, (ii) Chemical compatibility with the structure of human bone [7]. Calcium-silicate-based ceramics exhibit biocompatibility, and *in-vivo* osteogenesis [8]. It acts as a potential applicant in bone renovation and regeneration. The main problem with calcium silicate is that the high pH can alter its strength and thus their utilization is restricted for some dental application [3]. Calcium-silicate-based ceramics, demonstrate antibacterial activity due to their alkaline property [9,10].

The significance of copper in proliferation and development is noticeable due to its extensive biochemical requirements in cellular processes. Copper, in its metallic and chemical compound forms, maintains the anti-inflammatory, anti-microbial, and anti-proliferative properties [11]. Cu^{2+} can be integrated into alloys, assuming that the released ions and the corresponding elevation in local pH will attain the desired antibacterial effect [12]. Ewald et al. showed that Cu^{2+} ions could improve the cell activity and proliferation of osteoblastic cells directly by mixing the Cu^{2+} ion solution with calcium phosphate [$Ca_3(PO_4)_2$] cements [13]. Earlier studies emphasized that the direct mixing of Cu^{2+} ions with bioactive materials is a feasible way to improve the angiogenesis process [14]. It is expected that the incorporation of Cu^{2+} into the biomaterials scaffolds can offer them additional antibacterial property. The mechanism of the antibacterial possessions may be attributed to, that copper can assist as an electron donor/acceptor by interchanging between the redox states of cuprous (Cu+) and cupric (Cu^{2+}) ions. Redox properties of copper can cause prospective damage to bacteria. In addition, the redox reaction can produce hydroxyl radicals [15].

MATERIALS AND METHODS

Synthesis of the sample

The production of copper (Cu^{2+}) doped $CaSiO_3$ was prepared via the wet precipitation method. For this purpose, [$CaCO_3$, 99%] from El-Gomhorya Company**,** Egypt, silica gel [SiO_2, Fluka] and copper carbonate [$CuCO_3$, Fluka] were engaged as precursors for Ca^{2+}, SiO_2 and Cu^{2+} respectively. Analytical nitric acid (HNO_3) was used during the experiment. In the present study, five

individually weight% concentrations of the Cu^{2+} [15-19] over /100 g of the $CaSiO_3$ solution were produced the preparation route was mentioned in previous work [20] according to the following reactions:

Powder characterization of the prepared batches

Powdered samples calcined at 550°C in air for 2 hours were subjected to X-ray diffraction (XRD) in order to detect the phase purity and crystallinity of the prepared composites. X-ray investigation of the individual and mixed solids was conducted using a XRD, Bruker, D8 Advanced Cu target, Germany.

The functional groups in the synthesized materials were analyzed using Fourier Transform Infrared Spectra (FTIR, model FT/IR-6100 type A, USA) between 400-4000 cm^{-1}.

The morphology and size of the samples were characterized via SEM coupled with energy-dispersive spectroscopy EDAX, [SEM Model Quanta FEG 250, Holland, samples were sputtered with gold]. Transmission electron microscope (TEM), [JEM-HR-2100, Japan] operated at an accelerating voltage of 200 Kv was used. The aqueous dispersion of the particles was drop-casted onto a carbon-coated copper grid then, air dried at room temperature before being microscopically screened.

In-vitro antibacterial and antifungal activities

Anti-bacterial activity

Qualitative evaluations were carried out in nutrient agar disc and ciprofloxacin was used as standard according to Mostafa et al., 2016 [21]. Bacteria used in this study were: Gram positive bacteria (*Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC29213) and Gram negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27953). The inoculation of all microorganisms was prepared from fresh overnight broth cultures that were incubated at 37°C [18]. The spore suspension of pathological strains was prepared and adjusted to approximately (1×10^8 spores-ml). 1 ml of spore suspensions was inoculated into each plate containing 50 ml of the sterile nutrient agar medium (NA). After the media cooled and solidified, 100 mg of the prepared powder samples were applied on the inoculated agar plates and incubated for 24 hours at 30°C. Growth of the inhibition zone (IZ) was recorded after 24 hours of incubation at 37 ± 2°C [22]. The diameter of inhibition zone formed around the specimens was measured in (mm) at three different points and the average value was reported as Mean ± SD using the MS Excel.

Anti-fungal activity

Antifungal activity was evaluated against pathogenic fungi (*Aspergillus niger* NRC53 and *Fusarium solani* NRC15) by the agar diffusion technique [17]. 1 ml of spore suspension (1×10^6 spores-ml) was inoculated into each plate containing 50 ml of sterile potato dextrose agar (PDA). 100 mg of the powder samples were applied on the inoculated agar plates after cooling and incubated for 72 h at 28°C. The inhibition zone diameter around samples was measured in mm [4].

RESULTS

X-ray diffraction patterns (XRD) exposed the composition osf the prepared samples as revealed in Figure 1. It shows the crystallization of quartz (82-1574 Card № SiO_2), wollastonite (76-1846 Card № $CaSiO_3$) and Ca-olivine (80-942 Card № Ca_2SiO_4) in Cu-free samples and those containing CuO up to 3.0%.

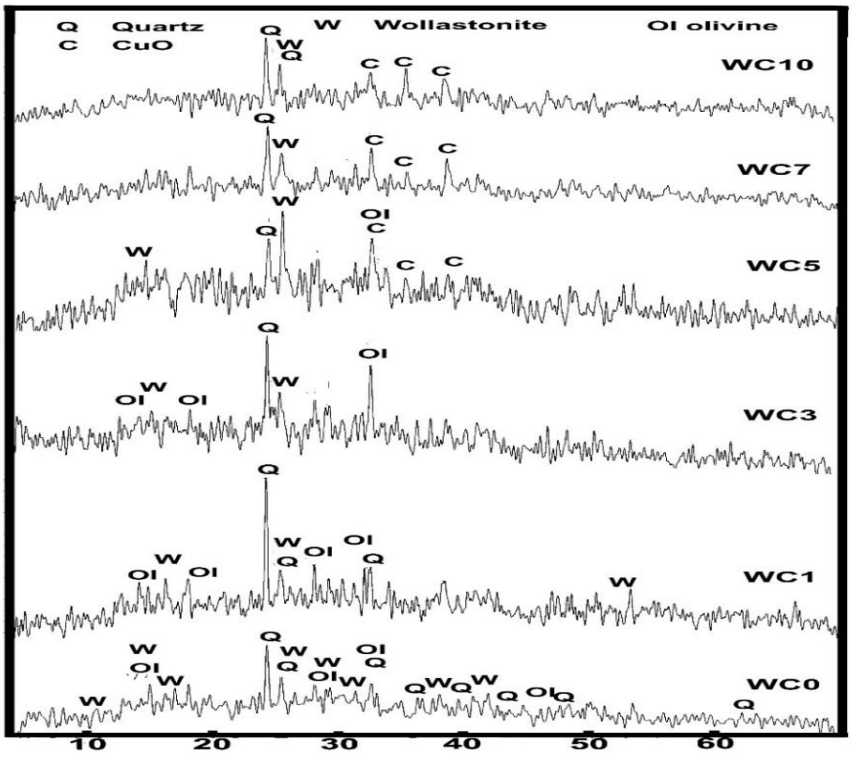

Figure 1: XRD patterns of blank and different concentrations of CuO doped samples after calcination at 550°C/2h.

However, incorporation of more than 3.0% CuO caused the development of tenorite (CuO) phase in addition to the later phases which became the highest in case of the 10.0% CuO sample Figure 1.

FTIR transmittance of the blank, 3.0% and 5.0% CuO powder shows various bands (Figure 2). The vibration bands between 2130-3419 and that at 3932 cm^{-1} may be attributed to the stretching vibrations of O-H groups in the H_2O with a wide range of hydrogen bond strengths [1]. Band at 1637 cm^{-1}, is due to H-O-H bending vibration of the molecular H_2O [23]. The previous band may improve the bioactivity of the produced composites [9]. Moreover, bands at 1428-1442 cm^{-1} assigned to carbonate disappeared from samples doped with 3.0%, 5.0% and the blank ones. FTIR spectra display the characteristic vibrational modes occurring at wave numbers 474, 563, and 593 cm–1 for the 3.0% CuO, which may be corresponding to the vibrations of Cu-O. While, the 5.0% CuO elucidates the peaks at 476, 559, and 592 cm^{-1} confirming the formation of highly pure CuO NPs [24]. Both samples showed a moderate band around 1025 cm^{-1} which could be ascribed to the CuO phase. These results coincide with the values available in literature [25].

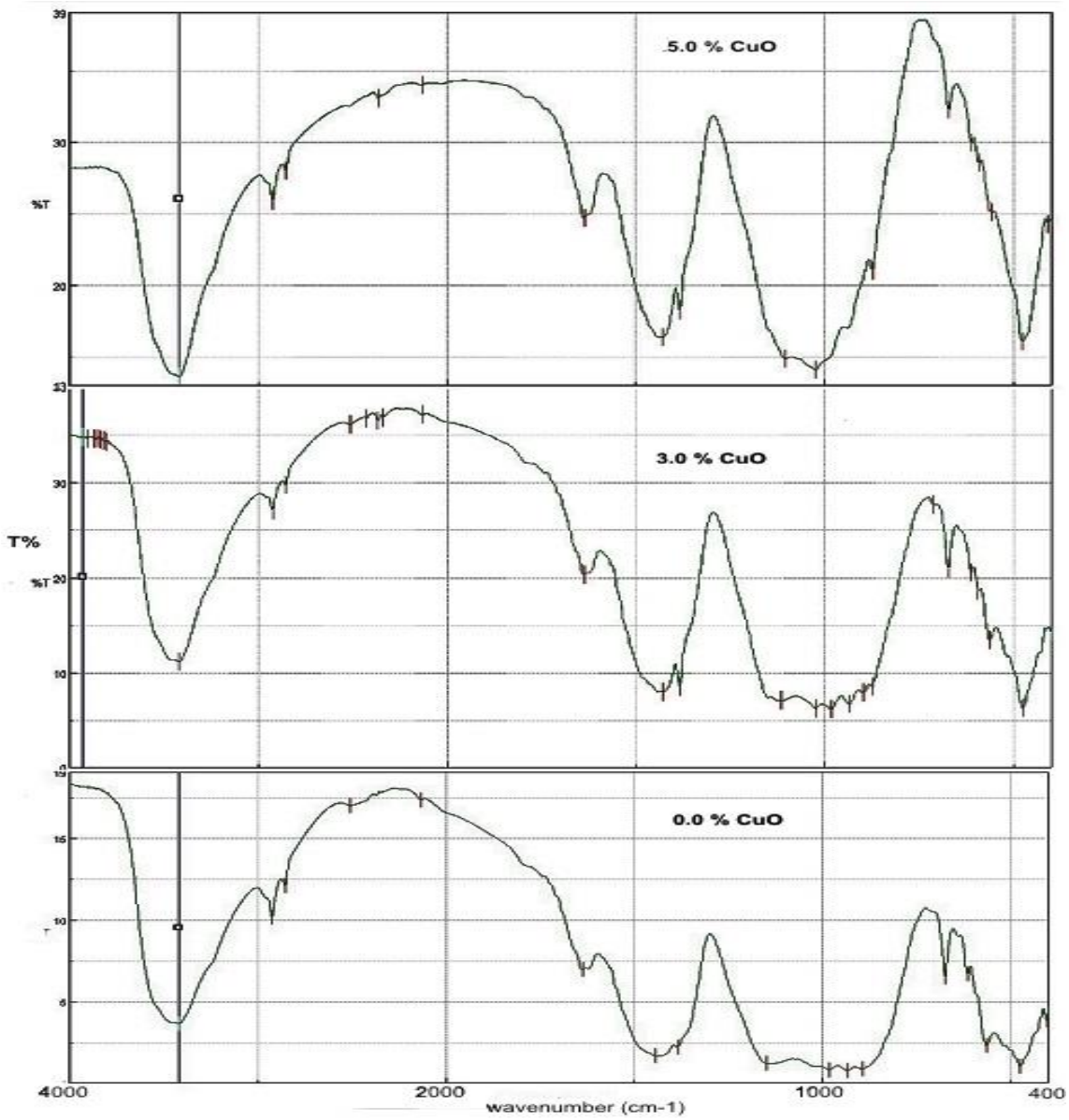

Figure 2: FTIR analysis of the blank, 3 & 5% CuO doped powder after calcination at 550^{o}C

TEM displayed rounded to sub rounded grains in the nano-scale size about 50 nm (Figure 3). This was confirmed by the SEM micrographs of both the blank and 5.0% CuO (Figure 4) Blank sample showed irregular agglomerated particles, in a size ranging from 40-50 (nm).

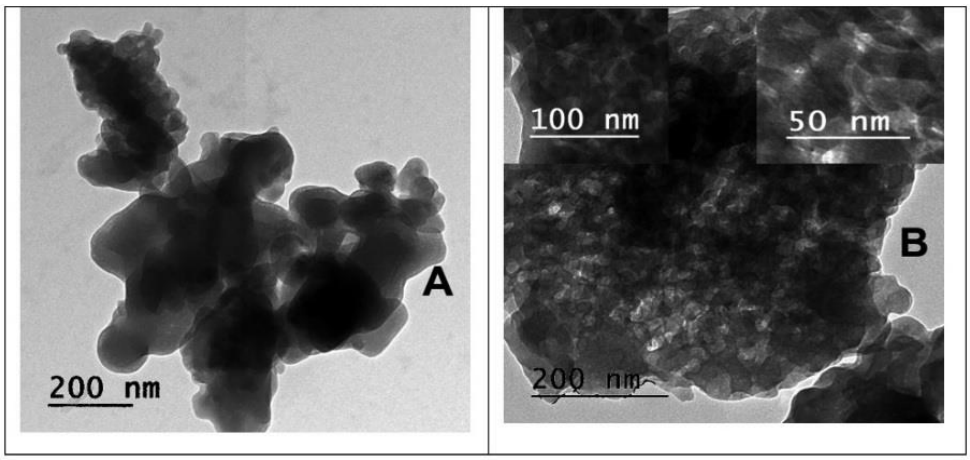

Figure 3: TEM analysis of: **(A)** blank and **(B)** 5.0% CuO samples after calcination at 550°C/2h.

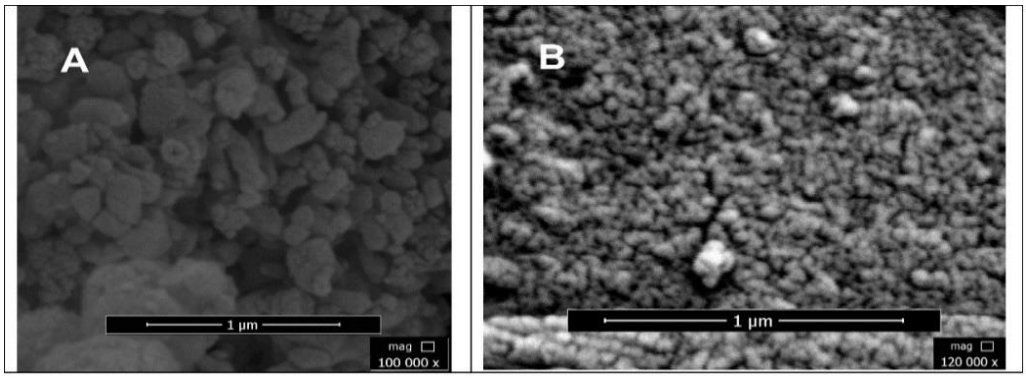

Figure 4: SEM images of: **(A)** blank **(B)** 5.0% CuO after calcination at 550°C/2h.

Alternatively, the 5.0% CuO sample revealed interconnected fine particles in the range of 19-31 (nm). EDAX microanalysis of 5.0% CuO sample confirms the incorporation of Cu into the wollastonite structure (Figure 5).

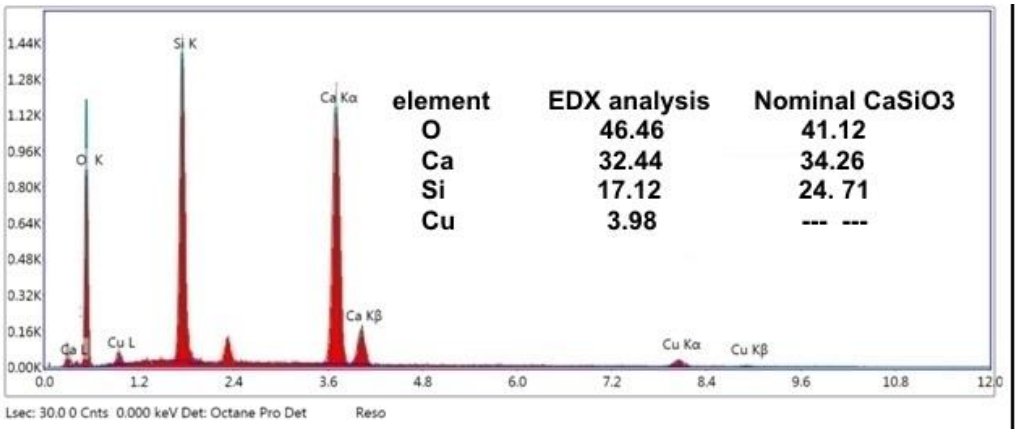

Figure 5: EDAX microanalysis of the 5% CuO after calcination at 550°C/2h

Antimicrobial assay photos and IZD of the samples against pathogenic bacteria and fungi were presented in Figure 6 and Table 1. All different concentrations displayed a wide range of antibacterial activity against both *Gram positive* and *Gram negative bacteria*, with inhibition zones varying from 14-30 mm. Furthermore, they showed good antifungal activity against both *F. solani* and *A. niger* with inhibition zone diameter (IZD) in the range of 15-22 mm. Results demonstrated that calcium silicate doped with copper enhanced the activity. *P. aeruginosa* and *A. niger* were found to be most susceptible to the 5.0% CuO with IZD of 30 and 22 mm, respectively. The Gram negative *E. coli* showed the widest IZD of 20 mm in case of the 3.0% CuO. Other concentrations revealed almost similar inhibitory activity against the Gram positive bacteria in the range of 14 -18 mm.

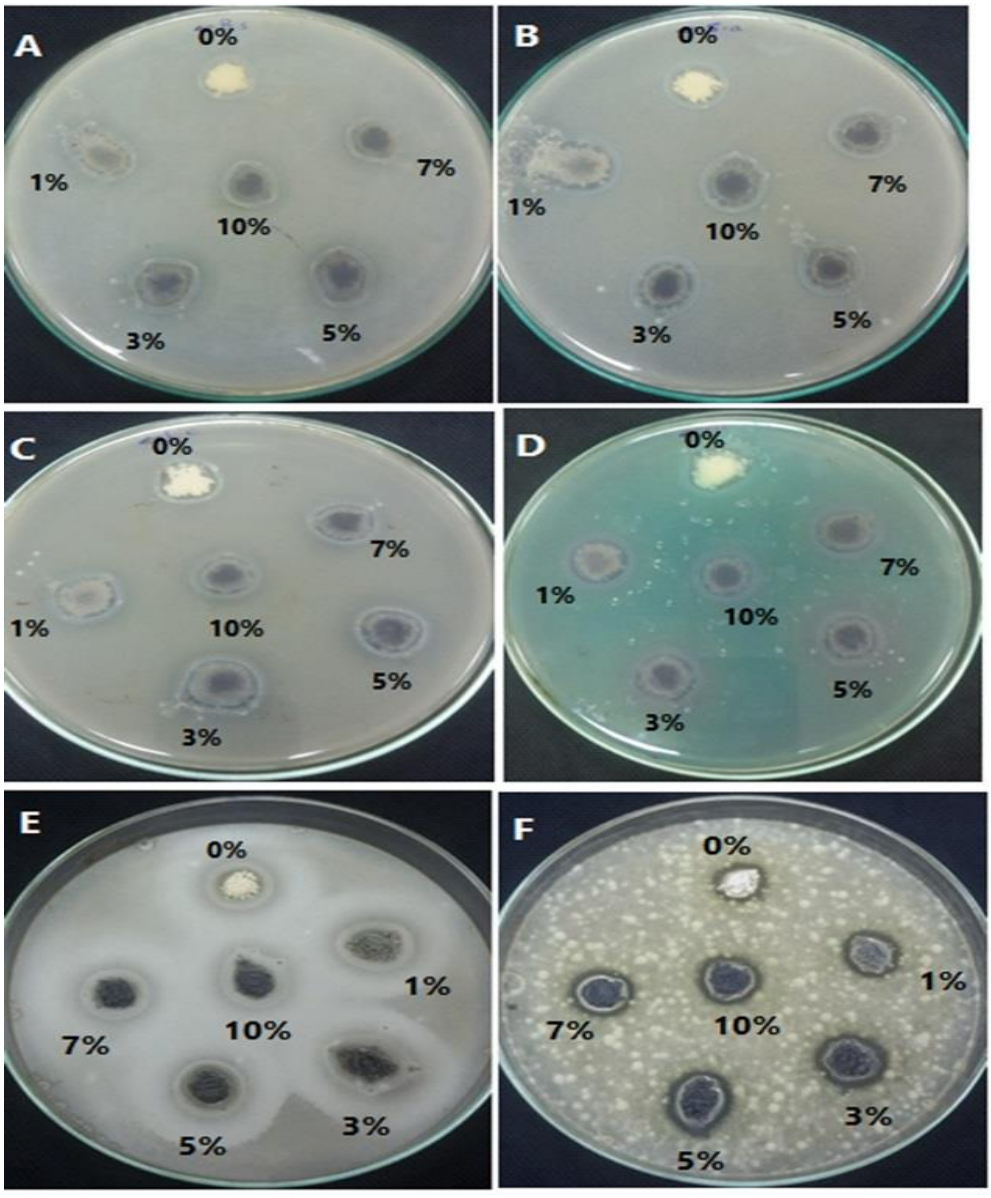

Figure 6: Anti-microbial activity of the 5% CuO as-prepared sample compared with blank.

Table 1: Inhibition zone diameter of calcium silicate and calcium silicate doped with Copper

Sample	Inhibition zone diameter (IZD (mm)					
	Gram positive bacteria		Gram negative bacteria		Fungi	
	B. subtilis ATCC6633	***S. aureus ATCC29213***	***E. coli ATCC 25922***	***P. aeruginosa ATCC27953***	***F. solani NRC18***	***A. niger NRC53***
WC0	13 ± 0.70	15 ± 0.70	17 ± 0.70	17 ± 2.12	11 ± 1.41	16 ± 0.70
WC1	17 ± 2.24	18 ± 2.12	17 ± 0.70	18 ± 0	18 ± 2.12	20 ± 1.41
WC3	17 ± 1.41	18 ± 1.41	20 ± 0.70	25 ± 0	18 ± 3.53	21 ± 0.70
WC5	17 ± 0.70	16 ± 0.70	18 ± 0.70	30 ± 0	16 ± 1.41	22 ± 2.94
WC7	15 ± 0.70	18 ± 2.12	19 ± 1.41	24 ± 0	15 ± 0.70	20 ± 0.70
WC10	14 ± 0.70	18 ± 2.82	17 ± 0.70	23 ± 0	17 ± 0.70	20 ± 0

No significant trend was noticed in the case of the inhibitory action against *S. aureus*. Results verified that Gram negative bacteria *Pseudomonas aeruginosa* (ATCC27953) and fungi *Aspergillus niger* (NRC53) were found to be most susceptible to the 5.0% CuO with inhibition zone diameter (IZD) of 30 mm and 22 mm, respectively. The Gram negative *Escherichia coli* (ATCC25922) showed the top IZD of 20 mm in case of 3.0% CuO (Figure 7).

On the other hand, the stability test of the most effective samples [3.0% and 5.0% CuO] was presented in Figures 8 and 9. This highlights good resistance to most of the tested strains along 8 days. However, the 3.0% CuO powder sample showed good resistance to *P. aeruginosa* and moderate resistance to *B. subtilis*, *E. coli*, *A. niger* and had weak stability against *S. aureus* and *F. solani* (Figure 8). Stability test of the 5.0% CuO powder showed good resistance to most of tested strains along 8 days (Figure 9).

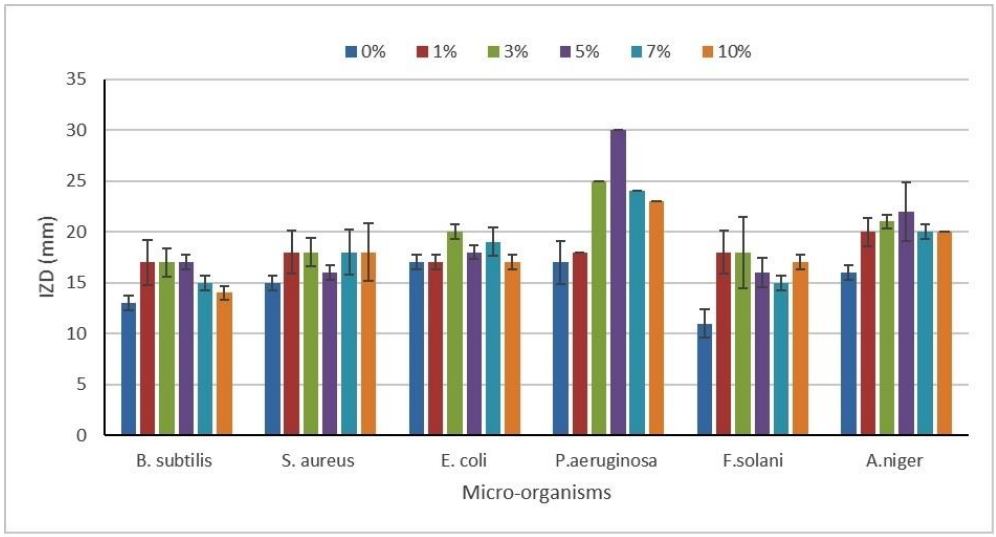

Figure 7: Effect of calcium silicate doped with 3 and 5% CuO on the inhibition zone diameter of Gram negative bacteria *Pseudomonas aeruginosa* (ATCC27953) and fungi *Aspergillus niger* (NRC53).

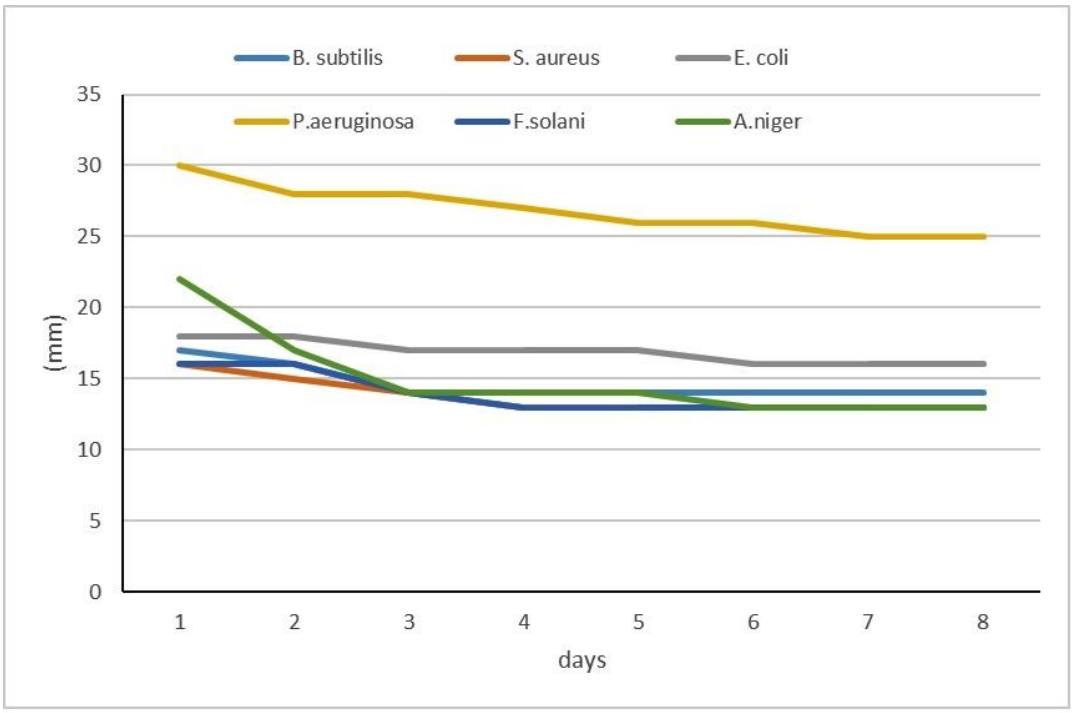

Figure 8: Effect of 3% CuO powder sample on resistance of *P. aeruginosa* and *B. subtilis, E. coli, A. niger*

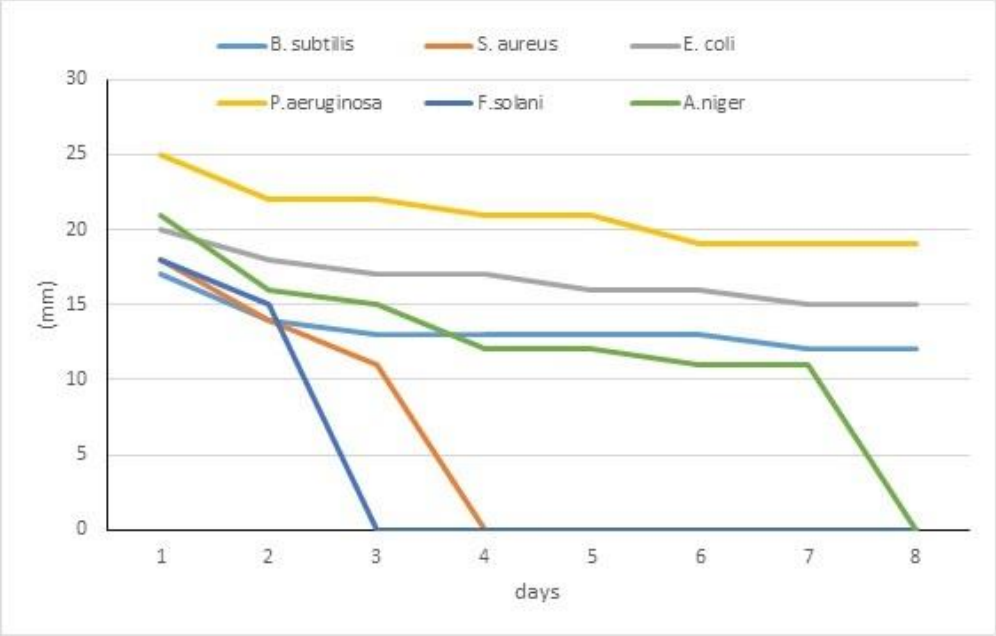

Figure 9: Emphasized the efficiency of the prepared wollastonite powder with CuO against pathogenic bacteria and fungi

DISCUSSION

The FTIR spectra presented the characteristic vibrational modes occurring at wavenumbers 474 cm^{-1}, 563 cm^{-1} and 593 cm^{-1} for the 3.0% CuO, which may be corresponding to the vibrations of Cu-O. Whereas samples doped with 5.0% CuO showed peaks at 476 cm^{-1}, 559 cm^{-1} and 592 cm^{-1} showing the formation of highly pure CuO NPs **[**26**].** These findings are in alignment with the values available in literature [27]. The typical morphology (TEM) was confirmed by the SEM micrographs in both the 0.0 and 5.0% CuO samples. EDAX microanalysis of the 5.0% CuO sample indicates the incorporation of copper into the wollastonite structure. There was a gradual increase in the inhibition zone diameter (IZD) with an increase in the amount of Cu^{2+} content. While, for the 0.0% copper concentration the least inhibitory effect was shown in comparison with the other samples indicating the importance of the addition of copper as an antimicrobial agent. It was observed that doping of copper up to 5 mol% improved the inhibition zones against *V. Cholerae and E. coli* pathogens during the antimicrobial study [28**].** Kaur et al., found that additional increase in the content of copper from 3-5 mol% decreases the cell viability of the samples, which emphasized that the clusters containing 3.0% CuO are effective on *E. coli* [26].

It is worthy to indicate that the anti-bacterial activity of bioactive glass samples was explained via a minimum inhibitory zone formation test**.** [29] stated that despite the well-known anti-bacterial action of silver (Ag), a narrower inhibition zone was observed compared to the copper doped samples **[**29-31**].** This could be justified on basis of dissolution behavior of ions from the doped bioactive glass samples. The degree of susceptibility of the tested micro-organisms to samples containing copper can be arranged as the following order *P. aeruginosa* > *A. niger* > *E. coli* > *S. aureus* > *F. solani* > *B. subtilis*. Results emphasized the efficiency of the prepared wollastonite powder with CuO against pathogenic bacteria and fungi.

CONCLUSION

Based on wollastonite, rounded to sub rounded clusters were prepared by wet method doped with/without CuO. The clusters were quantitatively tested on Gram negative bacteria (*Pseudomonas aeruginosa and Escherichia coli), Gram* positive bacteria (*Bacillus subtilis and Staphylococcus aureus*) and Fungi (*Aspergillus niger and Fusarium solani*) using agar diffusion technique. The CuO-containing samples showed good degree of susceptibility against *P. aeruginosa, A. niger fungi and E. coli respectively*. The clusters containing 5.0% CuO showed inhibition zone diameter (IZD) in the range of 30 and 22 mm, respectively. Other clusters containing 3.0% CuO showed moderate IZD (20 mm. Clusters of wollastonite with/without CuO prepared via wet precipitation route can be considered a novel antimicrobial agent for bone implantation.

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