Study of the Antimicrobial Activity of Cuprite Synthesized by Chemical Route

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With the rise of nanotechnology, inorganic nanomaterials have been developed which have a marked microbicide effect on a wide variety of microorganisms such as viruses, bacteria and fungi. In the present work, the synthesis, characterization and study of the antimicrobial activity of cuprite (copper (I) oxide, Cu_2O), obtained by chemical route was carried out. To obtain cuprite, copper sulfate pentahydrate was used as a precursor and ascorbic acid and glucose as organic reductants. The synthesis using glucose as a reductant resulted in the obtaining of 100% cuprite with variable morphology, spheres, cubes and tetrahedra were observed, very dependent on the concentration of NaOH used in each synthesis, with particle sizes in the nanoscale and micrometer, that is, nanoparticles and Cu_2O nanostructures were obtained. According to the results of the antimicrobial activity, it can be concluded that Cu_2O copper oxide has an antimicrobial effect on Staphylococcus aureus bacteria, with the Minimum Inhibitory Concentration, MIC, 16 mg/mL.

Keywords: cuprite, antimicrobial, Staphylococcus aureus

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that is widely distributed in the environment because it has particular characteristics of virulence and resistance against antibiotics, which represents a serious health problem, in addition this bacterium has genetic characteristics that have allowed it to become one of the most important bacteria in hospitals and in foodborne diseases. According to the literature, copper and copper oxides are known to be used in different sanitary and medical equipment for their bactericidal and antimicrobial properties. The mechanism that explains the antibacterial activity is the ability of copper to continuously give up and accept electrons. Copper ions are released from copper surfaces penetrating into the bacterial cell causing rupture of the cytoplasmic membrane favoring the entry of copper ions, leading to membrane dysfunction and increased oxidative stress. At the cytoplasmic level there is an alteration of protein synthesis and functional damage of essential enzymes leading to cell death and degradation of bacterial DNA. Although copper may cause the bacteria to lose the ability to replicate by altering the molecular structure, it is not known to cause mutations in the DNA. Any of these mechanisms affects the survival of bacteria, but it will depend on the concentration of the metal to see the bacteriostatic or bactericidal effect. The Minimum Inhibitory Concentration (MIC), in microbiology, is the lowest

concentration of an antimicrobial that inhibits the growth of a microorganism after incubation. The minimum inhibitory concentration is important in laboratory diagnostics to confirm the resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents.

In the literature there are several works available on the synthesis of copper, CuO and Cu_2O, using organic reductants and surfactants whose results show that the synthesis by this chemical route is simple and most importantly allows the control of the size and shape of the synthesized particles. There are also publications regarding the antimicrobial activity of copper and copper oxides on various gram-positive as well as gram-negative bacteria, which generally suggest that Cu, CuO and Cu_2O nanoparticles can be considered as new effective agents of multidrug resistant bacteria. This research work aims to synthesize cuprite, Cu_2O, from the precursor salt $CuSO_4.5H_2O$ and using ascorbic acid, glucose and hydrazine as organic reductants, taking advantage of the fact that the use of organic reductants requires low temperatures during the synthesis process. To perform the microstructural characterization of the synthesized particles through X-Ray Diffraction Analysis (XRD) and Scanning Electron Microscopy (SEM) and finally to study the microbicidal activity of cuprite on Staphylococcus aureus bacteria in order to determine the minimum inhibitory concentration (MIC).

EXPERIMENTAL SECTION

For the synthesis of copper oxide, we started from the precursor in the form of copper sulfate pentahydrate ($CuSO_4.5H_2O$), which was reduced with ascorbic acid ($C_6H_8O_6$) in acid medium and with glucose ($C_6H_{12}O_6$) in basic medium.

Synthesis Using Ascorbic Acid as a Reducing Agent

We proceeded to weigh 5.03 g of copper sulfate pentahydrate and 12.01 g of ascorbic acid. Then the precursor salt and the organic reductant were dissolved at room temperature using distilled water. 18mL of distilled water was added to the 5.3g of $CuSO_4*5H_2O$ and 30mL of distilled water to the 12.01g of ascorbic acid. After dissolving the salts separately, the solution containing the copper sulfate pentahydrate was added little by little to the container containing the ascorbic acid solution, stirring vigorously. The formation of a dark green final solution with a pH of approximately 1.66 was observed. After finishing the addition of all the copper sulfate pentahydrate solution to the ascorbic acid solution, the final solution was heated to a temperature of 70°C for 20 min using a heating plate with magnetic stirring. The formation of an orange precipitate was observed at the base of the beaker, which gave us indications that it was pure copper. The supernatant solution was decanted and the beaker was washed several times with distilled water. The sample was finally washed with alcohol, dried, packaged and labeled.

Synthesis Using Glucose as Reductant

5.71g of copper sulfate pentahydrate was weighed and made up to 50ml in a beaker containing distilled water. 2.13g of glucose and 3.78g of sodium hydroxide were weighed and made up to 25ml each in their respective beakers.

Initially, sodium hydroxide was added to the beaker containing glucose. The formation of a slightly yellowish solution was observed. To this yellowish solution we added little by little the copper sulfate pentahydrate that was placed in a burette. As $CuSO_4.5H_2O$ was added, it changed from blue to dark green. The solution was constantly stirred throughout the process and the change in pH at each addition of copper sulfate from the burette was noted. As the pH increased, the solution was observed to become darker, until a reddish precipitate was observed at pH 12.3. When the titration was finished, the beaker containing the precipitate was heated on a heating plate at 60°C for 30 minutes. The supernatant solution was then decanted to discard it and the precipitate was washed 5 times with distilled water and a final wash with alcohol. Finally, the precipitate was dried at less than 50°C. Once the sample was dry, the final weight was determined, and it was 1.47g.

The final samples were sent to external laboratories for X-Ray Diffraction (XRD) and Scanning Electron Microscopy (SEM) analysis.

RESULTS AND DISCUSSION

Analysis of the XRD Diffractograms of the Sample Synthesized Using Ascorbic Acid as Reductant in Acid Medium

The qualitative analysis shows that the peaks of the diffractogram of this sample coincide with the peaks corresponding to pure copper, as can be seen in Figure 1**.** Therefore it can be assumed that from the reduction of copper sulfate with ascorbic acid at pH= 1.66 100% pure copper is obtained, as was established in the work of Liu King Ming et al (2012), where they conclude that at very low pH pure copper of larger size (micrometric) is obtained and at high pH cuprite of smaller sizes (nanometric) is obtained, they also establish that the possible mechanism of formation of pure copper at pH greater than 2.5 is through the following stages: $Cu^{2+} \rightarrow Cu(OH)_2 \rightarrow Cu_2O \rightarrow Cu$, i.e. Cu^{2+} ions are initially transformed to $Cu(OH)_2$ and then $Cu(OH)_2$ is reduced to Cu_2O by ascorbic acid. Cu_2O is finally reduced to Cu particles, thus establishing Cu_2O as an intermediate product and representing the reduction process through the following reactions.

$$Cu(OH)_2 + C_6H_8O_6 \rightarrow Cu_2O + C_6H_6O_6 + H_2O \quad (1)$$

$$Cu_2O + C_6H_8O_6 \rightarrow Cu + C_6H_6O_6 + H_2O \quad (2)$$

In order to observe the possible routes of the copper sulfate reduction process in an aqueous medium, we can use the HSC 6 software to construct the Pourbaix Diagram of the Cu-O-H system at 60°C and for the Cu concentration in solution, which is 1.14 molal, as shown in **Figure 2.** It can be clearly observed that for pH equal to 1.66, which was the one used in the synthesis with ascorbic acid as reductant, pure copper can be synthesized in a single step.

FIGURE 1
COMPARATIVE ANALYSIS OF THE STANDARD DIFFRACTOGRAM OF COPPER AND THE DIFFRACTOGRAM OF THE EXPERIMENTAL SAMPLE OBTAINED FROM THE REDUCTION OF COPPER SULFATE WITH ASCORBIC ACID

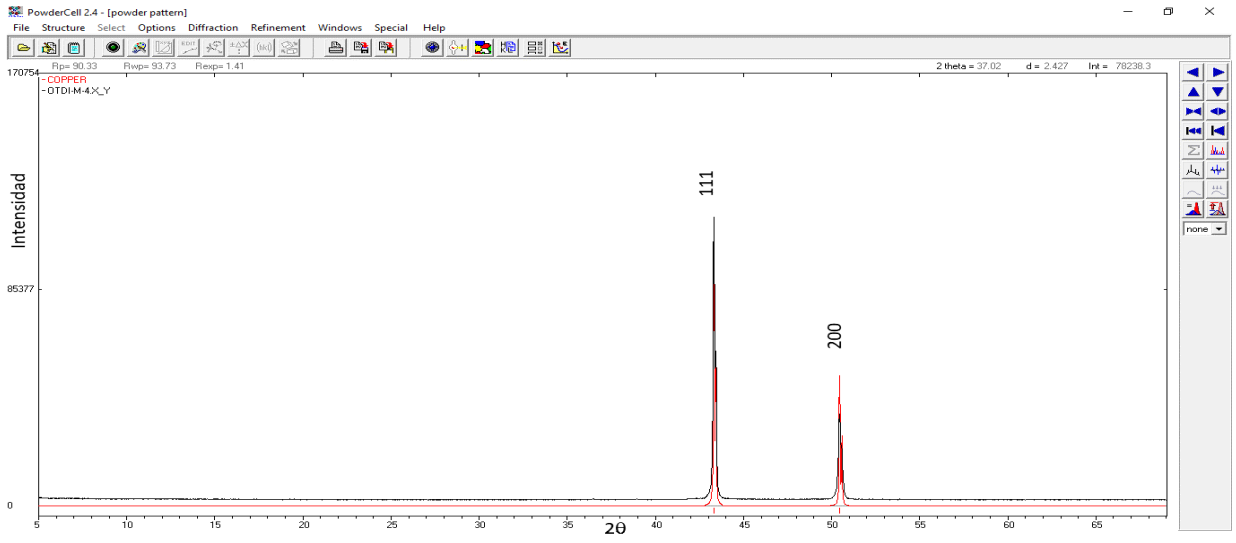
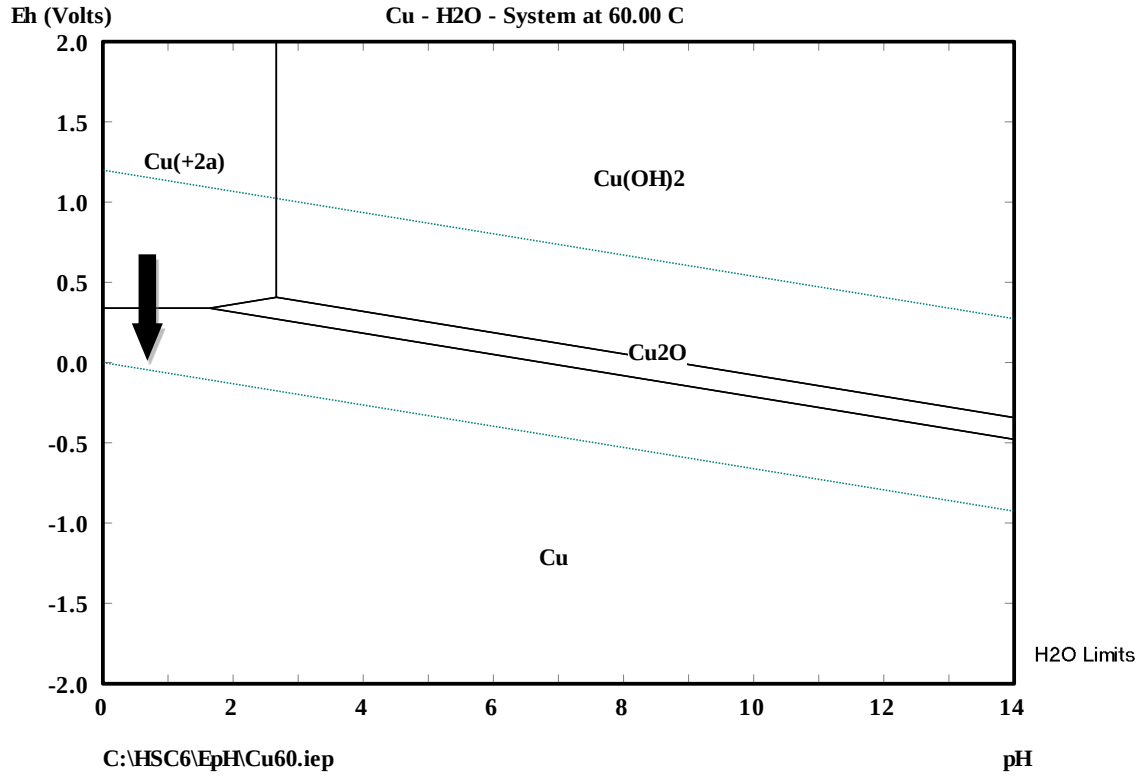


FIGURE 2
POURBAIX DIAGRAM OF THE CU-O-H SYSTEM AT 60°C AND FOR A COPPER CONCENTRATION OF 1.14 MOLAL, OBTAINED WITH HSC 6 SOFTWARE



Analysis of the XRD Diffractograms of the Sample Synthesized Using Glucose as Reductant in Basic Medium

In previous work using glucose and hydrazine as reductants, it was found that depending on the pH range employed in the synthesis, it is possible to obtain a metal/oxide mixture, i.e. Cu/Cu_2O, both in the case of using glucose as reductant and also hydrazine. When comparing the diffractograms of the experimental sample with the copper and cuprite diffraction patterns, it can be seen that the sample obtained using glucose as reductant at pH=12.3 presents copper and cuprite peaks, as shown in **Figure 3**. Quantitative analysis determined that the final sample contained 93.5% cuprite and 6.5% copper with a crystallite size of 83.23 nm. Some researchers established that Cu^{2}+ ions are first reduced to Cu^{+} by glucose and then the Cu^{+} reacts with OH^{-} to form Cu_2O, according to the following equations:

$$Cu(OH)_2 = Cu^{2+} + 2OH^- \quad (\text{or } CuO + H_2O = Cu^{2+} + 2OH^-) \tag{3}$$

$$Cu^{2+} + 3OH^- + C_6H_{12}O_6 = Cu^+ + C_6H_{11}O_7 + 2H_2O \tag{4}$$

$$Cu^+ + OH^- = CuOH \tag{5}$$

$$2CuOH = Cu_2O + H_2O \tag{6}$$

According to the previous results, it could be concluded that the synthesis using glucose as reductant could lead us to obtain 100% cuprite and therefore experimental tests were carried out by **modifying the pH**, i.e. varying the amount of NaOH used during the synthesis process of the sample. When making the

comparative analysis of the experimental and cuprite diffractograms, it is observed that in this case only the cuprite peaks appear, therefore 100% cuprite was obtained, **Figure 4**.

FIGURE 3
COMPARATIVE ANALYSIS OF THE STANDARD DIFFRACTOGRAMS OF COPPER AND CUPRITE WITH THE DIFFRACTOGRAM OF THE EXPERIMENTAL SAMPLE OBTAINED FROM THE REDUCTION OF COPPER SULFATE WITH GLUCOSE AT PH=12.3

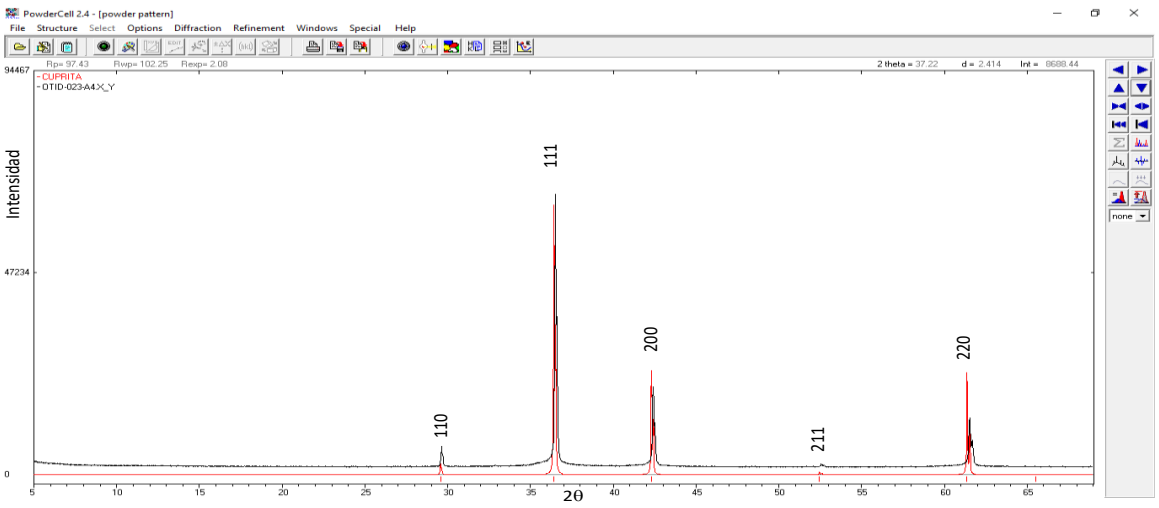
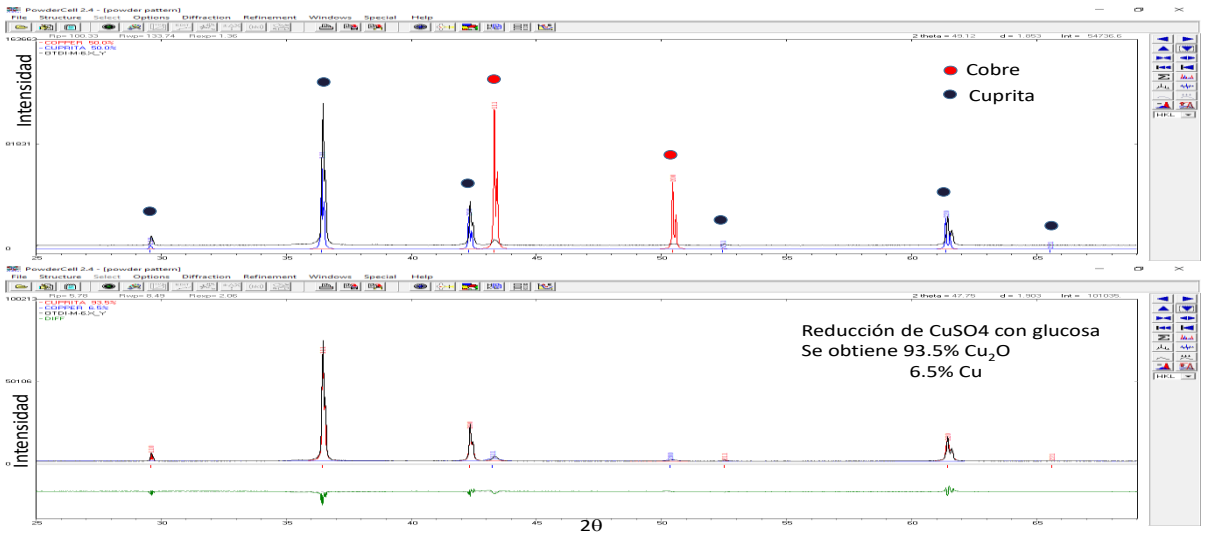
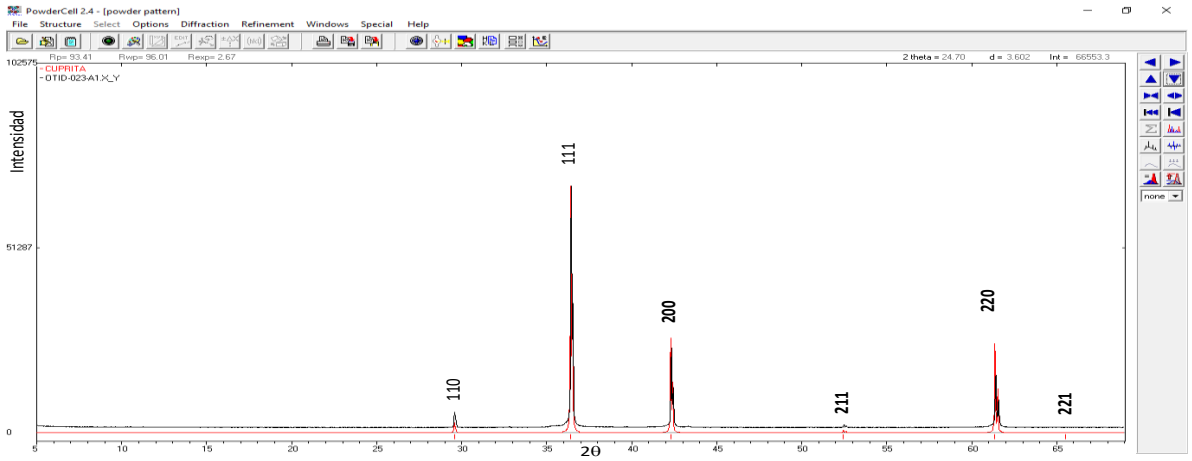


FIGURE 4
COMPARATIVE ANALYSIS OF THE STANDARD DIFFRACTOGRAM OF CUPRITE AND THE DIFFRACTOGRAM OF THE EXPERIMENTAL SAMPLE OBTAINED FROM THE REDUCTION OF COPPER SULFATE WITH GLUCOSE AT PH=12



Another modification that was made during the synthesis process using glucose as reductant was to perform the cuprite synthesis cold, i.e. without heating. When performing the comparative analysis of the experimental diffractogram with the standard diffractogram of cuprite, it can be seen that 100% cuprite was obtained, as can be seen in **Figure 5**.

FIGURE 5
COMPARATIVE ANALYSIS OF THE STANDARD DIFFRACTOGRAM OF CUPRITE AND THE DIFFRACTOGRAM OF THE EXPERIMENTAL SAMPLE OBTAINED FROM THE REDUCTION OF COPPER SULFATE WITH GLUCOSE AT PH=12.3, COLD SYNTHESIS



Analysis of SEM Micrographs of the Copper Sample Obtained Using Ascorbic Acid as Reductant in Acid Medium

The copper sample was sent to the Scanning Electron Microscopy Laboratory, School of Sciences of the National Engineering University. It can be seen in **Figure 6** that the particles are agglomerated and have an almost homogeneous size distribution, showing faceted crystals.

In **Figure 7** at higher magnification it can be clearly seen that the pure copper crystals are micrometric polyhedral particles averaging between 3 - 6µm in size. This is in agreement with what is found in the literature, which states that as the pH increases the particle size tends to decrease, in our case the experimental pH was 1.66, which is a very low value and therefore larger particles are obtained.

In other words, from the reduction of copper sulfate using ascorbic acid and at a pH of 1.66, particles in the shape of micrometer-sized polyhedrons are obtained.

FIGURE 6
SEM MICROGRAPHS AT A MAGNIFICATION OF 770X OF THE SAMPLE OBTAINED USING ASCORBIC ACID AS REDUCTANT IN ACID MEDIUM (PH=1.66)

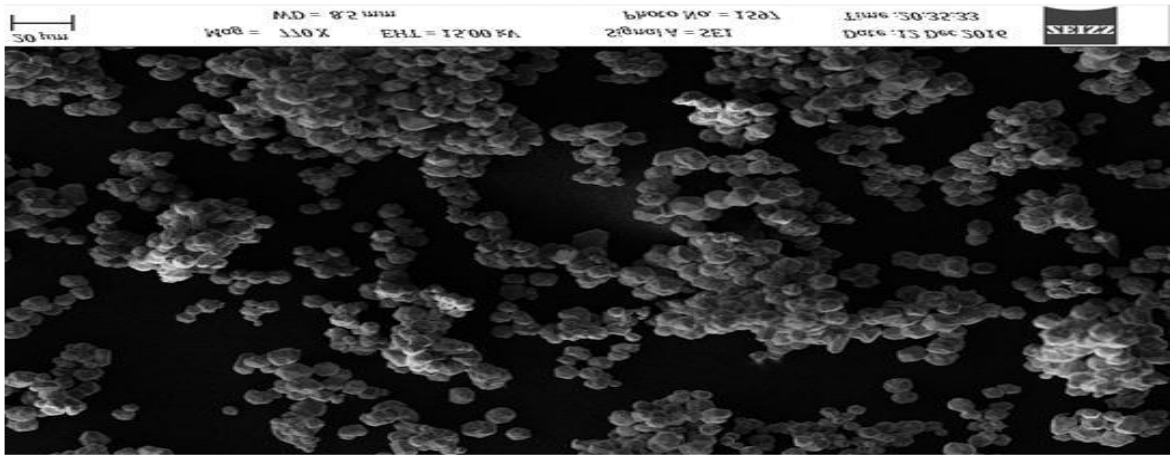
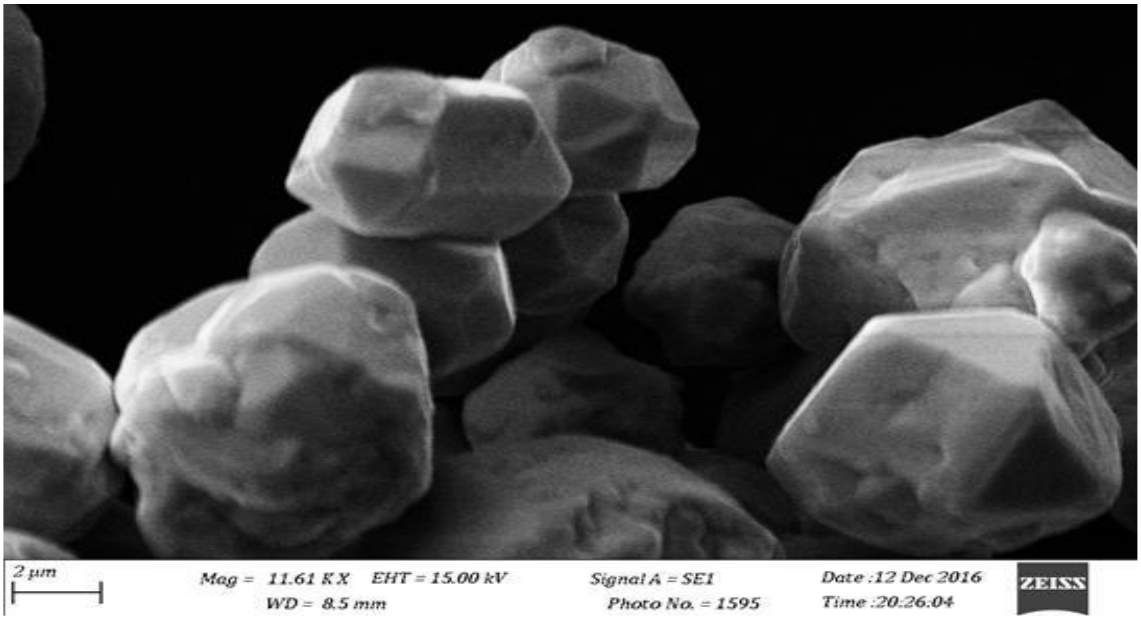


FIGURE 7
SEM 11.61 KX MICROGRAPHS OF THE COPPER SAMPLE OBTAINED FROM THE PRECURSOR COPPER SULFATE AND ASCORBIC ACID AS REDUCTANT



SEM Micrographs Analysis of Cuprite Synthesized With Glucose at pH=12 and Heating

In **Figure 8** the SEM micrograph of the cuprite sample synthesized with glucose at pH equal to 12 and heating is shown. It can be seen that the particle morphology is spherical and with a heterogeneous size distribution. These SEM micrographs were obtained using the PUC-Rio Tapletop Scanning Electron Microscope.

This same sample was analyzed in the Scanning Electron Microscope of the School of Sciences of the UNI in order to have micrographs with higher magnification. In **Figure 9** it can be seen that at a magnification of 112.20 KX it is possible to synthesize cuprite particles of nanometric size, i.e. nanoparticles. Unfortunately, we did not have access to a Transmission Electron Microscope, TEM, to achieve higher magnification and have more detail of the samples.

SEM Micrographs Analysis of Cuprite Synthesized With Glucose at pH=12.3 and Heating

In **Figure 10** the SEM micrographs at 5KX, 10KX, 20KX and 30KX of the cuprite sample synthesized with glucose at pH equal to 12.3 and heating are shown. It can be seen that the particle morphology is a mixture of quasi-spherical, cubic and tetrahedral with a heterogeneous size distribution. Particles with tetrahedral morphology are the smallest with size less than 1um, quasi-spherical, cubic and some tetrahedral particles have an average size of 3um. It can be appreciated in the cubic particles of the micrograph at 30KX that the particle is made up of small crystallites. In addition, it can be observed that cubic particles migrate to quasi-spheres and then these to tetrahedrons.

FIGURE 8
SEM MICROGRAPHS AT 5KX, 10KX, 20KX AND 30 KX OF THE CUPRITE SAMPLE SYNTHESIZED AT PH=12 AND WITH HEATING

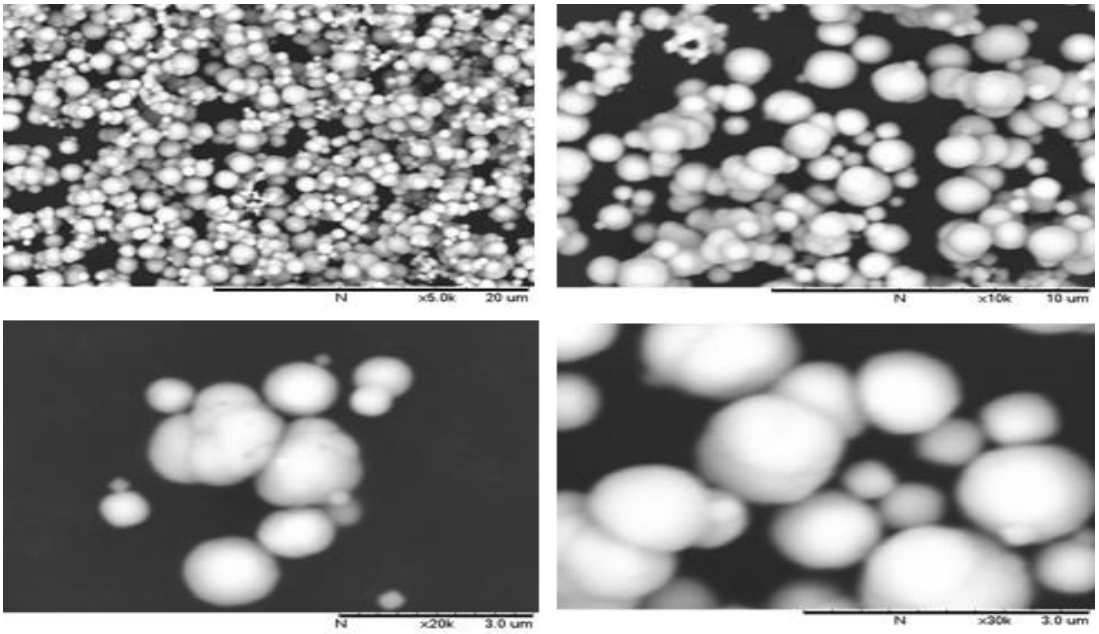


FIGURE 9
SEM MICROGRAPH AT 112.20 KX OF THE CUPRITE SAMPLE SYNTHESIZED AT PH=12 AND WITH HEATING

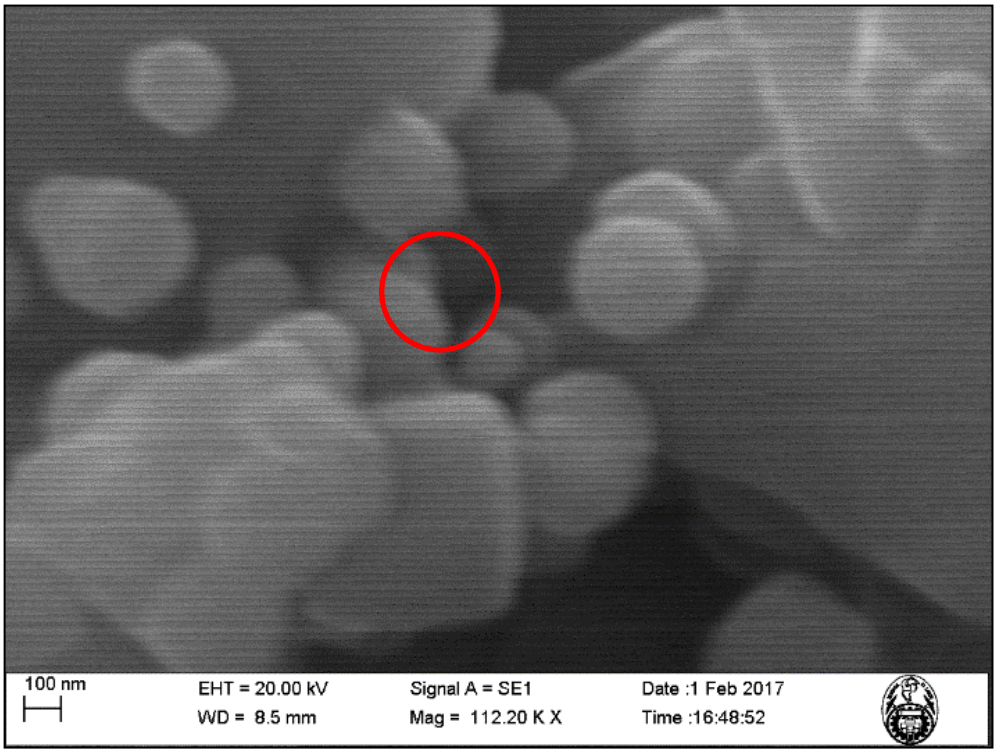
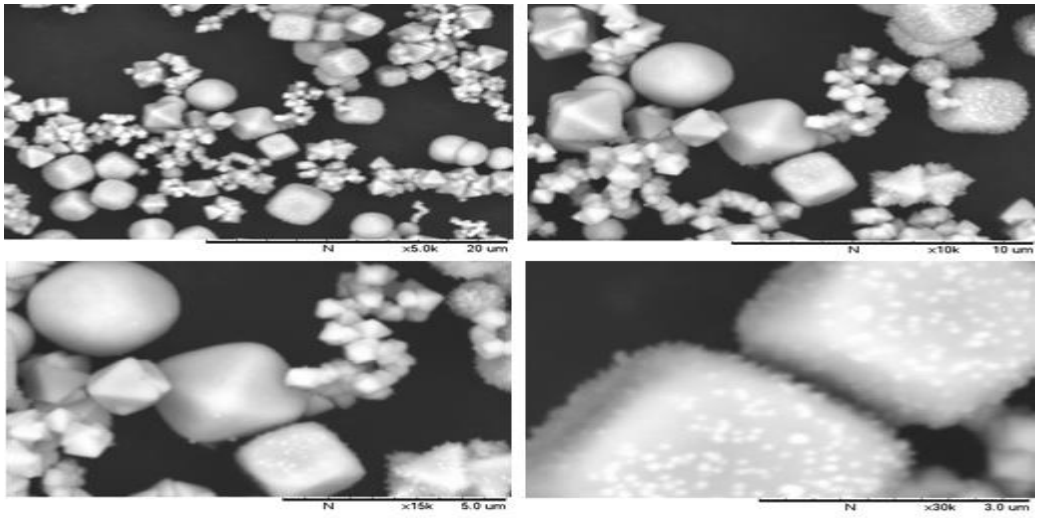


FIGURE 10
SEM MICROGRAPHS AT 5KX, 10KX, 20KX AND 30 KX OF THE CUPRITE SAMPLE SYNTHESIZED AT PH=12.3 AND WITH HEATING

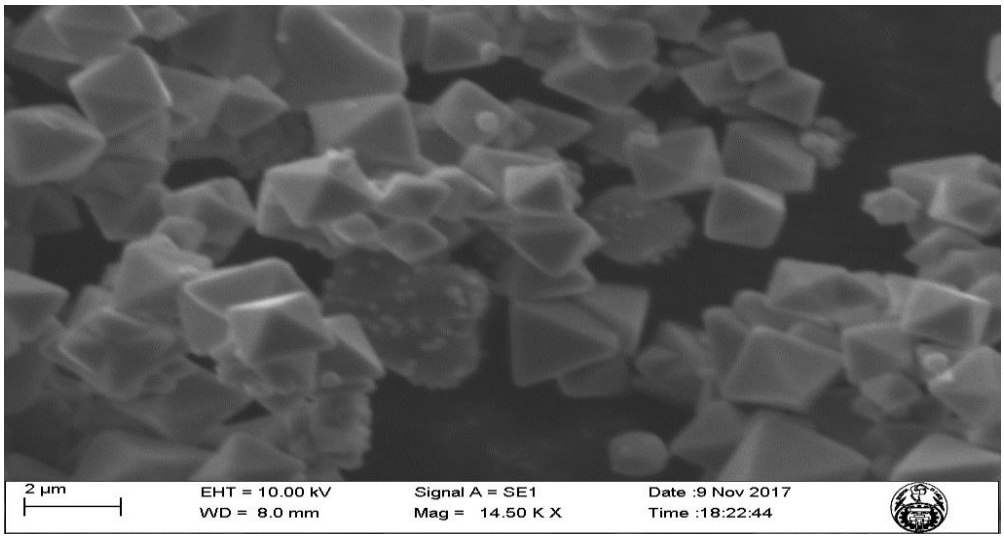


SEM Micrographs Analysis of Cuprite Synthesized With Glucose at pH=12.3 and Without Heating

In **Figure 11** the SEM micrograph at 14.50KX of the cuprite sample synthesized with glucose at pH equal to 12.3 and without heating, i.e. cold synthesized, is displayed. It can be seen that the particle morphology is tetrahedral with a fairly homogeneous size distribution.

According to the results obtained we can conclude that cuprite of different morphologies can be synthesized by reducing Cu(II) with glucose at 60°C or in cold and alkaline medium. It was discovered that the shape of Cu_2O particles changes with the modification of NaOH concentration, i.e. with the variation of pH. The different shapes of Cu_2O particles are caused by the absorption of OH^- ions in Cu_2O particles, which arise in the variety of Cu_2O growth mode, and then influence the final morphology of Cu_2O particles.

FIGURE 11
SEM MICROGRAPHS AT 14.50 KX OF THE CUPRITE SAMPLE SYNTHESIZED AT PH=12.3 AND WITHOUT HEATING



Analysis of the Assay Results for the Determination of the Minimum Inhibitory Concentration, MIC

In the Minimum Inhibitory Concentration (MIC) assays, an ATCC 43300 Staphylococcus aureus strain resistant to methylcyclicin was used. The culture medium used for strain growth and MIC was Muller Hinton. The cuprite particles were dissolved in this medium and the MIC reading was performed by spectrophotometry at a wavelength of 600nm. The initial strain concentration followed Mac Farland's equivalence of 0.5.

Assay With Cuprite Sample (Glucose as Reductant)

In this assay, the concentrations of 144, 48, 16, 5.3, 1.7 and 0.56 mg/mL of the cuprite sample were taken. **Table 1** shows the volume in µL used in the preparation of the cuprite concentrations. The negative control corresponds to the concentration of 16 mg/mL.

TABLE 1
VOLUME IN µL USED IN THE PREPARATION OF CUPRITE CONCENTRATIONS IN THE CONCENTRATION RANGE 144-0.56MG/ML

	B	C^-	C^+	Copper Concentration (mg/mL)					
				144	48	16	5.3	1.7	0.56
MH	1500	1365	1400	185	995	1265	1355	1385	1395
Cobre		135		1215	405	135	45	15	5
Strain			100	100	100	100	100	100	100

The absorbance results after OD measurement are shown in **Table 2:**

TABLE 2
RESULTS OF THE CUPRITE SAMPLE ABSORBANCE

C-	C+	144 mg/mL (1)	48 mg/mL (2)	16 mg/mL (3)	5.3 mg/mL (4)	1.7 mg/mL (5)	0.56 mg/mL (6)
1.182	0.65	2.478	2.154	1.025	0.521	0.569	0.554
1.163	0.716	2.318	2.001	1.003	0.524	0.6	0.593
1.082	0.727	2.272	2.021	0.978	0.526	0.654	0.576
1.142	0.698	2.356	2.059	1.002	0.524	0.608	0.574

Figure 12 and Figure 13 show the staining after preparing the vials with the strain and the particle concentrations maintained until the end of the MIC. **Figure 12** shows the staining after vortexing the medium and **Figure 13** shows that the nanoparticles have sedimented after 20 minutes. As observed in the negative control, the staining affects the OD. The difference between the negative control with its counterpart (vial 3 = 16mg/mL) gives an OD of 0.304, so the MIC of the sample could be 16mg/mL.

FIGURE 12
IMAGE SHOWING THE STAINING OF THE VIALS AFTER VORTEXING

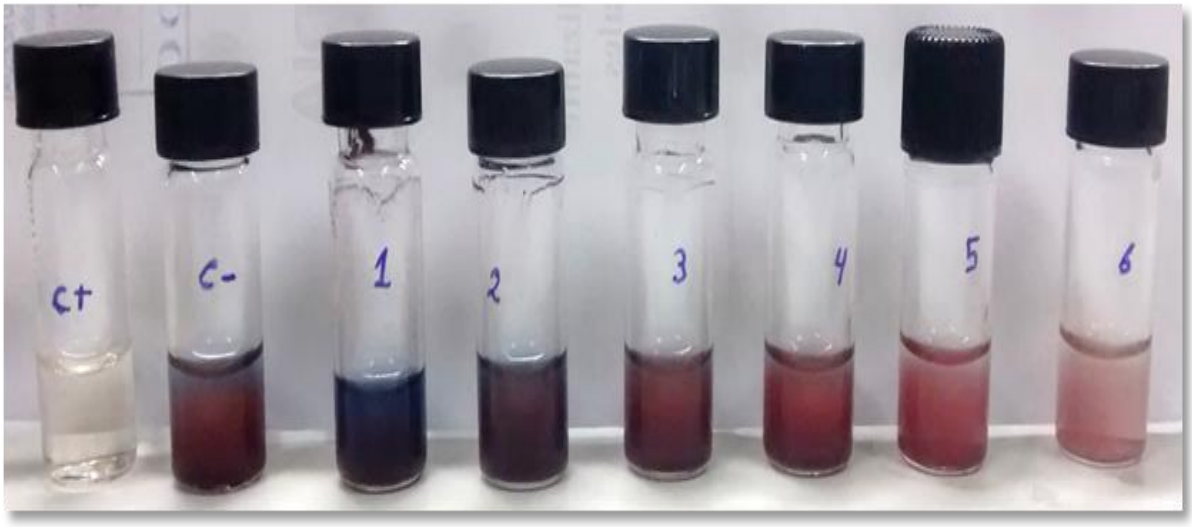
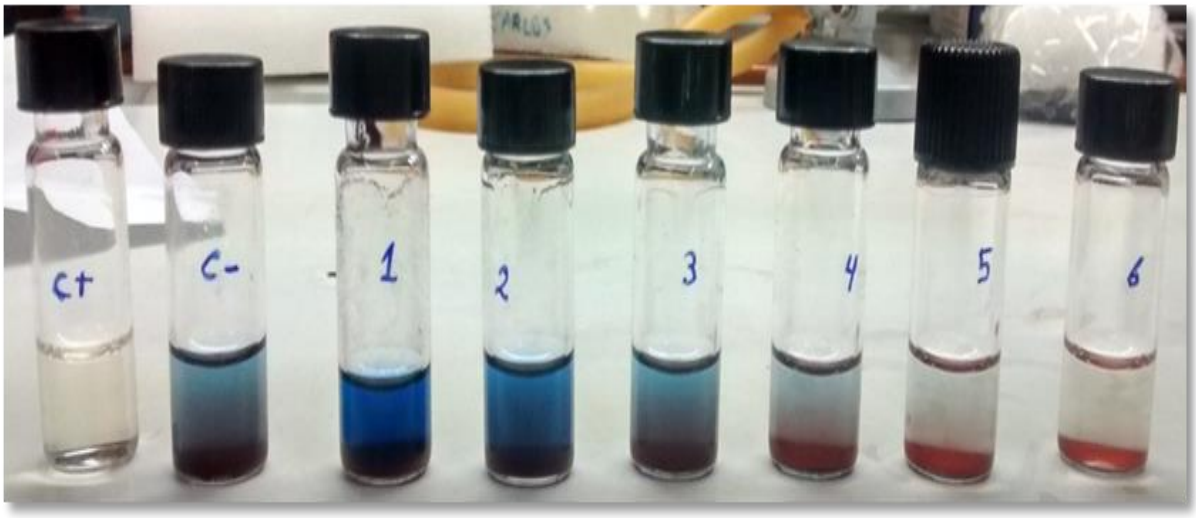


FIGURE 13
IMAGE SHOWING PARTICLE SEDIMENTATION AFTER 20 MIN OF VORTEXING

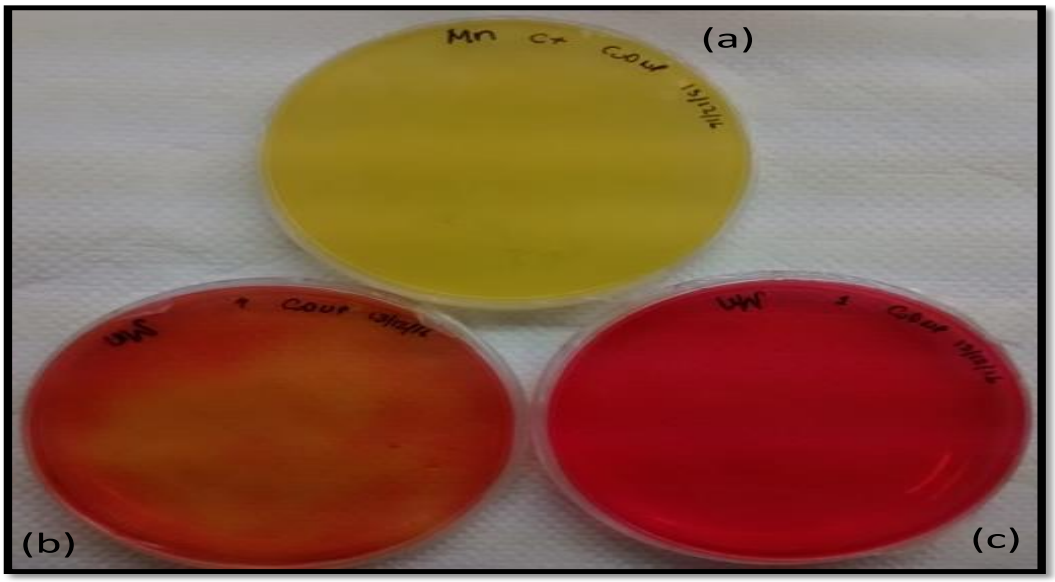


Bacterial Growth Test Results Analysis

Figure 14 shows the growth of the control strain (*Staphylococcus aureus*) in salted Mannitol medium in the Petri dish at the top. In the bottom left Petri dish, strain growth is observed where the concentration of vial 4 corresponds to 5.3 mg/mL, while in the bottom right Petri dish, no strain growth is observed at a concentration of 144mg/mL. Therefore the Minimum Inhibitory Concentration, MIC, corresponds to the concentration at which the plate showing no growth followed by the plate showing growth would be at a concentration of **16mg/mL**.

Therefore, this value of 16 mg/mL would be the Minimum Inhibitory Concentration, MIC, of cuprite, necessary to prevent the growth of *Staphylococcus aureus* bacteria, finally achieving the final objective of this research work.

FIGURE 14
IMAGE SHOWING BACTERIAL GROWTH



a) Growth of *Staphylococcus aureus* bacteria without the presence of cuprite. b) Partial growth of *Staphylococcus aureus* bacteria in presence of 5.3 mg/mL cuprite. c) Total inhibition of the *Staphylococcus aureus* bacteria growth in presence of 16mg/mL cuprite.

CONCLUSIONS

What is obtained from the synthesis of copper sulfate pentahydrate using ascorbic acid as reductant at a pH equal to 1.66 is 100% pure Cu, with a polyhedral morphology and micrometric size, while from the synthesis of copper sulfate pentahydrate using glucose as reductant and at a pH of 12.3, 93.5% Cu_2O and 6.5% Cu are obtained. The particles obtained under these conditions have the shapes of spheres, cubes and tetrahedrons, all of heterogeneous size.

100% cuprite can be obtained through modification of the NaOH concentration during synthesis, which affects pH, morphology and particle size. At pH=12, particles of spherical morphology are obtained. In addition, it is possible to obtain 100% cuprite by synthesis without heating, thus obtaining particles with tetrahedral morphology.

In connection with the antimicrobial activity of cuprite particles synthesized by chemical route using glucose as reductant in basic medium, it can be concluded that copper oxide, cuprite, Cu_2O, has an antimicrobial effect on the bacterium Staphylococcus aureus resistant to methylicin and that the Minimum Inhibitory Concentration, MIC, corresponds to a concentration of 16 mg/mL.

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