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Nanopartículas de Cobre y Óxido de Zinc; Propiedades Fisicoquímicas, Actividad Antimicrobiana y Aplicaciones Multifuncionales

Copper and Zinc Oxide Nanoparticles: Physicochemical Properties, Antimicrobial Activity and Multifunctional Application

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TÍTULO	**Copper and Zinc Oxide Nanoparticles: Physicochemical Properties, Antimicrobial Activity and Multifunctional Application**		
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PALABRAS CLAVE

Nanopartículas de cobre; Nanopartículas de óxido de zinc; Actividad antimicrobiana; Propiedades fisicoquímicas; Aplicación multifuncional.

PLANTEAMIENTO DEL PROBLEMA

Este artículo explora las principales propiedades de las nanopartículas de cobre y óxido de zinc con el objetivo de mejorar las condiciones sanitarias de los espacios públicos. El cobre (Cu) tiene alta conductividad eléctrica, alto punto de fusión, bajo comportamiento de migración electroquímica y bajo costo [1]. El óxido de zinc (ZnO) es un compuesto inorgánico ampliamente utilizado en aplicaciones cotidianas. Las nanopartículas de compuestos orgánicos presentan una fuerte actividad antibacteriana a bajas concentraciones debido a su gran área superficial y propiedades fisicoquímicas. Este artículo presenta los principales métodos de síntesis de las nanopartículas de Cu y ZnO, las principales propiedades fisicoquímicas y el efecto antimicrobiano en su incorporación a una comunidad bacteriana.

RESULTADOS

Es importante tener en cuenta que la muestra preparada con ZnO NP se llama K3. Por otro lado, las muestras preparadas con Cu NP se denominan K9 y K10. Echando un vistazo a la actividad antibacteriana de K3, se observa que durante períodos cortos de tiempo, la disminución de la concentración de las nanopartículas de ZnO conduce a un aumento de la actividad antibacteriana, mientras que durante largos períodos de tiempo al aumentar la concentración de estas nanopartículas, aumenta también la actividad antibacteriana. En la muestra K9, con nanopartículas de Cu, se obtiene que durante períodos cortos de tiempo, al aumentar la concentración de las nanopartículas, se da un aumento de la actividad antibacteriana y durante largos períodos de tiempo a medida que aumenta la concentración de estas nanopartículas, la actividad antibacteriana también aumenta. En la muestra K10 se obtiene que

durante períodos cortos de tiempo aumentando la concentración de nanopartículas de Cu, se obtiene que durante períodos cortos de tiempo, aumentar la concentración de estas partículas conduce a un aumento de la actividad antibacteriana, mientras que durante largos períodos de tiempo la concentración de al disminuir la concentración de las nanopartículas, la actividad antibacteriana aumenta.

CONCLUSIONES

La explicación del comportamiento de K3 es la presencia de materia orgánica en la muestra, que podría haber actuado como fuente de carbono para los microorganismos, alimentándolos. En este caso, la acción de ZnO no es lo suficientemente fuerte como para superar esta alimentación de los microorganismos. Por esta razón, cuando no hay suficiente tiempo para que ZnO neutralice esta alimentación, a mayor concentración de ZnO, aparecen una mayor concentración de microorganismos. Sin embargo, con el tiempo suficiente, las nanopartículas de ZnO pueden neutralizar esta alimentación e inhibir el crecimiento del microorganismo. La muestra K9 muestra el comportamiento teóricamente esperado en todas las muestras; al aumentar la concentración de nanopartículas de cobre, aumenta la actividad antibacteriana, independientemente del tiempo de contacto entre microorganismos y la suspensión. El comportamiento de K10 podría explicarse con la presencia de materia orgánica en la muestra, que podría haber actuado como fuente de carbono para los microorganismos, por lo que puede ser un alimento para ellos. En este caso, la acción del Cu no es lo suficientemente fuerte como para neutralizar esta alimentación de los microorganismos. Comparando ahora las tres muestras entre ellas, la conclusión principal es que la mejor muestra es K9, debido a su alta inhibición de la tasa de crecimiento de los microorganismos y, por lo tanto, a la alta eficiencia en la actividad antibacteriana. Además, la diferencia en K9 y K10 (ambos con nanopartículas de cobre) son las condiciones de síntesis; esa podría ser la razón de la diferencia en su actividad antimicrobiana. En términos generales, podemos concluir que, en este experimento, las nanopartículas de Cu han sido más eficientes que las de ZnO.

BIBLIOGRAFÍA

1. Tamilvanan, A., Balamurugan, K., Ponappa, K., and Madhan Kumar, B. (2014). Copper Nanoparticles: Synthetic Strategies, Properties and Multifunctional Application. *International Journal of Nanoscience,13* (2), 1430001. 22 pages.

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KEYWORDS

Copper nanoparticles; Zinc Oxide nanoparticles; Antimicrobial activity; Physicochemical properties; Multifunctional Application.

SCOPE

This article explores the main properties of Copper and Zinc Oxide nanoparticles with the aim of increasing the sanitary conditions of public spaces. Copper (Cu) have high electrical conductivity, high melting point, low electrochemical migration behavior and low cost[1]. Zinc oxide (ZnO) is an inorganic compound widely used in everyday applications. Nanoparticles of organic compounds in nanosized particles present strong antibacterial activity at low concentrations due to their high surface area and physicochemical properties. This review presents the main synthesis methods of Cu and ZnO nanoparticles, principal physicochemical properties and the antimicrobial effect on their incorporation to a bacterial community.

RESULTS

It is important to note that the sample prepared with ZnO NP's is named K3. On the other hand the samples prepared with Cu NP's are named K9 and K10, respectively. Taking a look in the antibacterial activity of K3, the sample with Zinc Oxide nanoparticles, it is seen that for short periods of time decreasing the concentration of ZnO NP's leads to an increase of the antibacterial activity, while for long periods of time as the the concentration of ZnO NP's increases, so does the antibacterial activity. In K9 sample, with Cu NP's, it is obtained that for short periods of time increasing the concentration of Cu NP's leads to an increase of the antibacterial activity and for long periods of time as the the concentration of Cu NP's increases, the antibacterial activity also increases. In K10 sample it is obtained that for short periods of time increasing the concentration of Cu NP's it is obtained that for short periods of time increasing the concentration of Cu NP's leads to an increase of the antibacterial activity, while for

long periods of time as the the concentration of Cu NP's decreases, the antibacterial activity increases.

CONCLUSIONS

One reason to explain the behavior in K3 is the presence of organic matter in K3 sample, which could have acted as a source of carbon for the microorganisms, so it can be a feed for them. In this case the action of ZnO is not strong enough for overcome this feeding properties of the composition. For this reason, when there is not enough time for ZnO to overcome this feeding, the Petris with higher concentration of ZnO NPs appear with higher concentration of microorganisms. However, for long periods of time, ZnO NP's are able to get over this feeding and inhibit the growth of the microorganism with zinc oxide nanoparticles. Sample K9 shows the theoretically expected behavior in all of the samples; increasing the concentration of Cu NP's leads to an increase of the antibacterial activity, regardless the time of contact between microorganisms and the suspension. The behavior of K10 could be explained with the presence of organic matter in K10 sample, which could have acted as a source of carbon for the microorganisms, so it can be a feed for them. In this case the action of Cu is not strong enough for overcome this feeding properties of the composition. Comparing now the three samples between them, the main conclusion is that the best sample is K9, because of its high inhibition of growth rate and therefore the high efficiency at antibacterial activity. Furthermore the difference in K9 and K10 (both having Copper nanoparticles) is the conditions of synthesis; that could be the reason of their difference in behavior, and therefore in their antimicrobial activity. In general terms we can conclude that, in these experiments, they have been more efficient Cu nanoparticles than ZnO nanoparticles.

REFERENCES

1. Tamilvanan, A., Balamurugan, K., Ponappa, K., and Madhan Kumar, B. (2014). Copper Nanoparticles: Synthetic Strategies, Properties and Multifunctional Application. *International Journal of Nanoscience,13* (2), 1430001. 22 pages

Research on innovative nanocomposites
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Copper and Zinc Oxide Nanoparticles: Physicochemical Properties, Antimicrobial Activity and Multifunctional Application

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ABSTRACT

This article explores the main properties of Copper and Zinc Oxide nanoparticles with the aim of increasing the sanitary conditions of public spaces. Copper (Cu) have high electrical conductivity, high melting point, low electrochemical migration behavior and low cost[1] . Zinc oxide (ZnO) is an inorganic compound widely used in everyday applications. Nanoparticles of organic compounds in nanosized particles present strong antibacterial activity at low concentrations due to their high surface area and physicochemical properties. This review presents the main synthesis methods of Cu and ZnO nanoparticles, principal physicochemical properties and the antimicrobial effect on their incorporation to a bacterial community.

Keywords: Copper nanoparticles; Zinc Oxide nanoparticles ; Antimicrobial activity; Physicochemical properties; Multifunctional Application.

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1. Introduction

Nanotechnology is the most promising technology that deals with understanding and control of matter nanoscale range. When a material is divided into small size particles in nanometer range, the individual particles exhibit unexpected properties which are different from those of the bulk material. Metallic nanoparticles find applications in optical, thermal, magnetic, sensoric devices and catalysis due to their small dimensions and special properties such as high surface area to volume ratio and high heat transfer[1].

Nowadays it is estimated that there are around 81 outbreaks and 1.326 cases of infections related to recreational waters (showers, changing rooms and common areas of swimming pools, spa, saunas or treatment rooms) in a year [2].

Therefore, it seems extremely important to continually improve the level of sanitary conditions at public bathing, saunas etc. However, increased amount of detergent used does not always provide a sterile environment. A disinfectant, e.g. chlorine may also threat the human health, since as a result of its reaction with organic substances chloramine which can cause inflammation of airway mucous membranes and respiratory tract. Chloramines are the product of the reaction of chlorine with the ammonium nitrogen and derivatives thereof. Their content is conditioned by the water pH and the stoichiometric ratio of free chlorine to ammonia nitrogen. However, organochlorine compounds such as

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chloroalkanes, carboxylic chloroacids or chlorophenols are more dangerous. These substances exhibit mutagenic and carcinogenic activity. Their presence in the air has been also confirmed, thus they may pose a constant threat to the staff. Chlorine causes allergies, atopic dermatitis and asthma, and is an irritating substance, so it should be treated in accordance with applicable regulations. For this reason, it is important to use the right technique that ensures sterile conditions.

Providing microbiological purity of water and recreational equipment while reducing the content of used disinfectant and products of their reactions is a serious technological problem. An excess of disinfectant may eliminate the risk of microorganisms growth, however it poses a threat to human health.

There are some proposed materials that contribute to increased antimicrobial safety. These materials, due to the presence of metals and metal oxide nanoparticles in their structure will exhibit biocidal properties, making them innovative materials for use in above-described conditions.

What is more, thanks to using nanomaterials which will be obtained using completely safe for the environment substances, the using of dangerous metallic nanoparticles whose properties threat the human body will be eliminated.

The group of compounds which are commonly used in obtaining nanomaterials include formaldehyde, hydrazine hydrate, sodium borohydride, hydrogen and polyvinylpyrrolidone, sodium dodecylsulfate, chitosan and ethylene glycol. However, the obtaining of nanometals using the above-mentioned compounds involves their negative impact on the environment. These are referred as toxic, irritating and harmful to the environment substances. Some of them are also carcinogenic and may have a particularly detrimental effect on aquatic organisms. Moreover, during the operation with above-mentioned substances it is necessary to adopt protective measures.

Hydrazine hydrate is a carcinogen substance of second category, which is highly toxic to aquatic organisms. Formaldehyde also exhibits toxic, irritating and corrosive properties. Sodium borohydride in contact with the skin releases flammable gases, causes burns and is one of highly flammable compounds. Aniline is defined as a toxic substance, which particulary reveals in contact with skin. Sodium dodecyl sulfate is commonly employed surfactant, which irritates the skin and pollutes water with sulfur compounds. Polyvinylpyrrolidone may contain unreacted vinylpyrrolidone mers which are carcinogenic, and ethylene glycol with air forms explosive mixtures, acts narcotic, causes damage to the central nervous system and spinal cord as well as irritates the mucous membranes. Thus, one of the main objectives of the project is to use the raw materials which do not endanger human health and the environment.

Therefore, the proposed project provides a double benefit: manufactured materials will have a biocidal effect and their physicochemical properties will not pose a threat to human health.

In the first phase of the project it is expected to obtain nanomaterials in the form of nanometals (Cu) and metal oxide nanoparticles (ZnO). The premise of this step is to carry out the synthesis of nanomaterials based on the principles of Green Chemistry and Cleaner Technologies.

The further general target of the project is to study the possibility of obtaining nanocomposites with antimicrobial properties for use them in facilities characterized by elevated moisture and temperature. In particular, it is assumed to obtain nanocomposites based on poly(vinylalcohol). Biocidal properties of these materials will be achieved through enrichment of their structure with previously obtained metal and metal oxide nanoparticles.

The aim of the project is also to define the characteristics of obtained materials in terms of their physicochemical properties, antimicrobial activity.

The analytic aim is to define dependences between physicochemical properties of obtained materials and set process parameters. The aim is also to assess the activity of obtained products in conditions that simulate its potential application environment, i.e. at elevated levels of temperature and humidity [3].

2. Synthesis of Copper and ZnO Nanoparticles

In the synthesis of these nanoparticles there are two key points to focus on; first of all, both kind of nanoparticles are prepared from precursors, not from pure substances. A precursor is a substance from which another compound is formed, specially by metabolic reaction. This is much more economic than using pure substances.

On the other hand, it is important to highlight that electrochemical potential expresses the stability of our nanoparticles in aqueous medium. This means that the more electrochemical potential the samples have, the more stable they are. In this review one of the objectives is to asses the values of electrochemical potential and determine if the samples studied are stable. The preparation of copper nanoparticles is much more difficult in comparison with noble metals due to the possibility of oxidation of copper with air. Copper nanoparticles have received much attention when compared to all other noble metals such as Ag, Au and Pt because of their small size, high surface/volume ratio, improvement of size, shape and oxidation resistance,etc [1].
The synthesis method used for Copper nanoparticles is chemical reduction. Liquid phase reduction is a chemical method to prepare copper nanoparticles. It involves precipitation of metallic Cu nanoparticles by chemical reduction of salt, oxide or hydroxide of copper in solution. In the chemical reduction method, a copper salt is reduced by using some reducing agent such as sodium borohydride, 26 potassium borohydride, 52 ascorbic acid, 53 hydra- zine54 and, tannic acid. Chemical reduction of copper salts is the most popular method because of its low cost and simple operation. Copper nanoparticles were produced with good control of size and morphology using chemical reduction of copper salts[1].
More specifically, the synthesis of copper nanoparticles of this experiment has been carried out with tannic acid as reducing agent. The mixture of these materials has the following chemical reaction equation:

$$Cu_2 + \text{tannic acid} \rightarrow Cu + \text{oxidized tannic acid}$$

The samples prepared with Cu nanoparticles are named K9 and K10, respectively.
They are both synthesized following the same steps; the first step is to weight 2,8 grams of PVA, add water and mix it in order to change PVA from solid to liquid form. Once the PVA is in liquid form (without sticky parts), the procedure consists on adding small amount of different compounds in the following order; Sacharoza, Hydroxyetylocelulose, Guar gum, Gelatine, Chitosan, Glycol, Copper Chloride * $2H_2O$, Tannic acid and Casein in NaOH. The material needed for the synthesis are, on one side, a mechanic stirrer for mixing constantly the compounds while they are added. On the other hand a heater is needed for maintaining the mixture on liquid state and facilitate the proper blended. The conditions of synthesis are more or less the same in both samples; the temperature on the heater should be between 60-80ºC depending on the sample. Furthermore, once all the compounds are added it is necessary to wait 30 min (K9) and 10 min (K10) for ensuring the blended is homogeneous and so that the copper nanoparticles are formed.
It is important to remark here that following the right order is crucial for the

synthesis. Otherwise, the copper nanoparticles would not be formed.

On the other hand, ZnO nanoparticles synthesis method is precipitation. In this method, the chemical reaction equation is the following:

$$Zn(NO_3)_2 + NaOH \rightarrow Zn(OH)_2 + NaNO_3$$

Knowing that;

$$Zn(OH)_2 \rightarrow ZnO + H_2O$$

For the synthesis of zinc oxide nanoparticles the first step is exactly the same; weighting 2,8 grams of PVA, add water and mix it in order to change PVA from solid to liquid form. Once the PVA is in liquid form (without sticky parts), the procedure consists on adding small amount of different compounds in the following order; Sacharoza, Hydroxyethyl Cellulose, Gelatine, Chitosan, Glycol, ZnO and Casein in NaOH.

The material needed for the synthesis of ZnO nanoparticles is, again, exactly the same as for Cu nanoparticles; on one side, a mechanic stirrer for mixing constantly the compounds while they are added.

On the other hand a heater is needed for maintaining the mixture on liquid state and facilitate the proper blended.

The conditions of synthesis change a little bit in comparison with the other synthesis; the temperature on the heater should be 60ºC. Furthermore, once all the compounds are added it is necessary to wait just 10 min for ensuring the blended is homogeneous and so that the nanoparticles are formed.

It is also necessary to maintain the right order, for the proper formation of the zinc oxide nanoparticles.

3. Physicochemical properties

Once we have the ZnO and Cu nanoparticles, it is time to characterize them in order to understand the control of the synthesis and their uses in wide applications. There are various techniques available for measuring the intrinsic properties of the nanoparticles. Microscopic techniques such as XRD, TEM, SEM, DLS, TEM, FTIR are commonly used to characterize the nanoparticles. Some of the important physical properties like size of particles, size distribution, crystallinity and morphology of nanoparticles were investigated using these techniques. The purpose of using various characterization techniques are given in Table 1[1][4].

Table 1 Summary of characterization techniques

TECHNIQUE	FACTORS STUDIED
X-RAY DIFFRACTION (XRD)	Structural characterization. Determination of crystal structure of unknown material and measurement of size and shape of unit cell.
SCANNING ELECTRON MICROSCOPY (SEM)	Information about the morphology, chemical composition, crystalline structure and orientation of materials of the sample.
FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)	Chemical changes in polymers after nanoparticle incorporation. Infrared spectrum of absorbance, emission and information of functional groups.
DYNAMIC LIGHT SCATTERING (DLS)	Also QELS. Measurements the size and size distribution of molecules and particles.
ENERGY DISPERSIVE X-RAY SPECTROSCOPY (EDS)	Used to identifiy the elemental composition of materials.

Once the characterization techniques are described, it is time to proceed with the pH measurement. The device used for the measurement is a pHmeter with a combined electrode, from Elmetron company. It is really important not only to measure the pH of the different samples but to relate this parameter with other physicochemical properties, e.g. particle size, in order to understand the relationship between the characteristics of the samples. Being aware and grasping this relationship would lead to a better understanding of the nanoparticles behavior.

Although there are two samples practically identical (K9, K10) and they theorically should have similar pH because both of them possess Copper nanoparticles, experimental results collected in Table 2 show that their pH are actually different. Both of the samples are acid, but K9 is much more acid that K10.

Table 2 Experimental results of pH measurements of each sample

	1	2	3	PH MEAN
K3 (ZNO)	6,76	6,75	6,76	**6,76**
K9 (CU)	4,5	4,45	4,45	**4,46**
K10 (CU)	6,8	6,82	6,88	**6,83**

The assessment of the density is another essential measurement for the posterior comprehension of the relationship between different characteristics of the samples.
Once we have set this relationship, it is possible to asses the way to improve the synthesis or the formation of the nanoparticles.

For the assessment of the density, the first step is preparing a tip of 5 ml with 1 ml of each sample. Then weight an empty beaker and finally weighting it again with the tip. Knowing that density is;

$$\rho = \frac{m}{V}$$

With
V= volume (1 ml in this case)
m= mass (the difference between empty beaker and full beaker in this case).

Table 3 Experimental results of density measurements of K3

K3	BEFORE [GRAMS]	AFTER [GRAMS]	DENSITY [G/CM^3]	AVERAGE DENSITY [G/CM^3]
1	35,007	36,484	1,477	
2	31,623	32,843	1,22	**1,32**
3	35,304	36,567	1,263	

Table 4 Experimental results of density measurements of K9

K9	BEFORE [GRAMS]	AFTER [GRAMS]	DENSITY [G/CM^3]	AVERAGE DENSITY [G/CM^3]
1	18,966	20,124	1,158	
2	31,885	33,141	1,256	**1,205**
3	34,396	35,597	1,201	

Table 5 Experimental results of density measurements of K10

K10	BEFORE [GRAMS]	AFTER [GRAMS]	DENSITY [G/CM^3]	AVERAGE DENSITY [G/CM^3]
1	16,651	17,820	1,169	
2	33,732	34,911	1,179	**1,164**
3	34,695	35,840	1,145	

It is seen that K10 has less density than K9, which could seem odd because both examples have copper nanoparticles. Later on, when we analyte the DLS results, we will try to relate this change in density with the particle size.

On the other hand, it is reasonable that K3 has more density than K9 and K10, because it has zinc oxide nanoparticles.

Once the density measurements are done, it is time to proceed with the structural analysis. Specifically with Dynamic Light Scattering (DLS). This technique measures the size of particles, the distribution of particle size and the electrochemical potential (also known as Zeta Potential, ζ). This kind of technique is based in the Mie Scattering, which describes the scattering of an electromagnetic plane wave by a homogeneous sphere. The term Mie theory is sometimes used for this collection of solutions and methods; it does not refer to an independent physical theory or law. More broadly, "Mie scattering" suggests situations where the size of the scattering particles is comparable to the wavelength of the light, rather than much smaller or much larger [5]. In other words, the measurement is made assuming that particles are spherical. For this study, it is necessary to introduce a drop of each sample in a tube-shaped container of 12 ml. Then add 10 ml of water. The apparatus used for DLS and Zeta Potential analysis is ZetaSizer Nanoseries, from Malvern Company. For the proper setting of the apparatus, it is necessary to introduce the refractive index of the different particles.

Table 6 Refractive index of ZnO and Cu nanoparticles

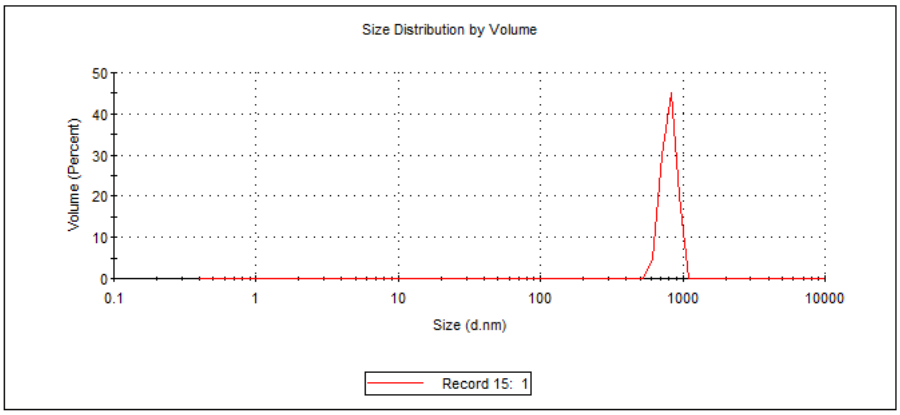
REFRACTIVE INDEX	ZNO	CU
	2,00	0,48

With the refractive index fixed , we can proceed with the analysis and obtain the results. Starting with the results of K3 (Figure 1).

Figure 1 Particle size and particle size distribution of K3

		Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 7050	Peak 1:	807,5	100,0	95,08
PdI: 0,291	Peak 2:	0,000	0,0	0,000
Intercept: 1,14	Peak 3:	0,000	0,0	0,000

Result quality : Refer to quality report



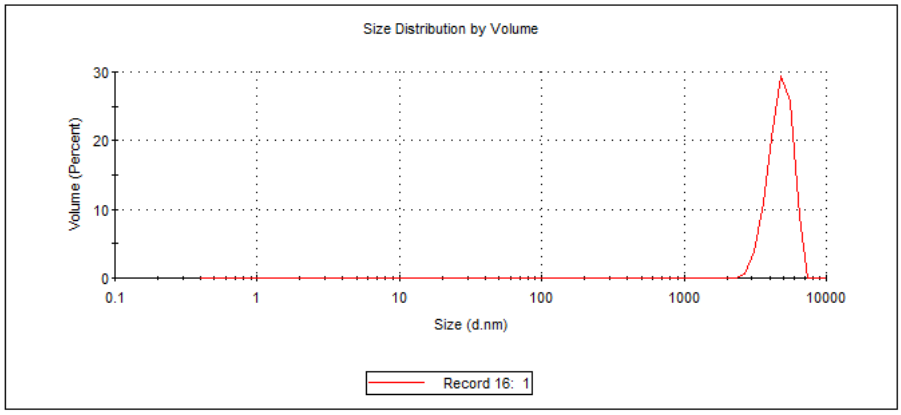
This particle size distribution curve is really narrowed, which means that all the particles have, more or less, the same size and volume. The size of the particles is approximately 808 nanometer, they are big nanoparticles, but the size result is reasonable.

Moreover, the absolute value of the electrochemical potential (also known as Zeta Potential) should be greater than 20 mV in order for the suspension to be stable. The Zeta Potential obtained for this suspension is -1,54 mV, which clearly means that the suspension is not stable.

Figure 2 Particle size and particle size distribution of K9

		Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 4738	Peak 1:	4796	100,0	884,5
PdI: 0,160	Peak 2:	0,000	0,0	0,000
Intercept: 0,954	Peak 3:	0,000	0,0	0,000

Result quality : Refer to quality report



This particle size distribution curve is a little bit wider than K3 curve, but still is narrow, so that the particles have the same size and volume. Here there was a problem, because the particle size seems to be 4796 nm, which is a very huge result.

This result is not acceptable. The solution proposed in order to know the size of the particles of K9 sample is to determine this size by the SEM-EDS results and compare them with this result. If the size obtained is the same as in DLS analysis, it means that this result is correct. Otherwise, this analysis should be repeated. The Zeta Potential is also really slow obtaining that it is -0,013 mV, which is really far away from the desired value of stability (20 mV), concluding that this suspension is not stable.

Figure 3 Particle size and particle size distribution of K10

		Size (d.nm):	% Volume:	St Dev (d.nm):
Z-Average (d.nm): 546,7	Peak 1:	636,6	6,5	152,1
PdI: 0,609	Peak 2:	57,81	2,7	17,82
Intercept: 0,986	Peak 3:	14,33	51,0	3,986

Result quality : Refer to quality report

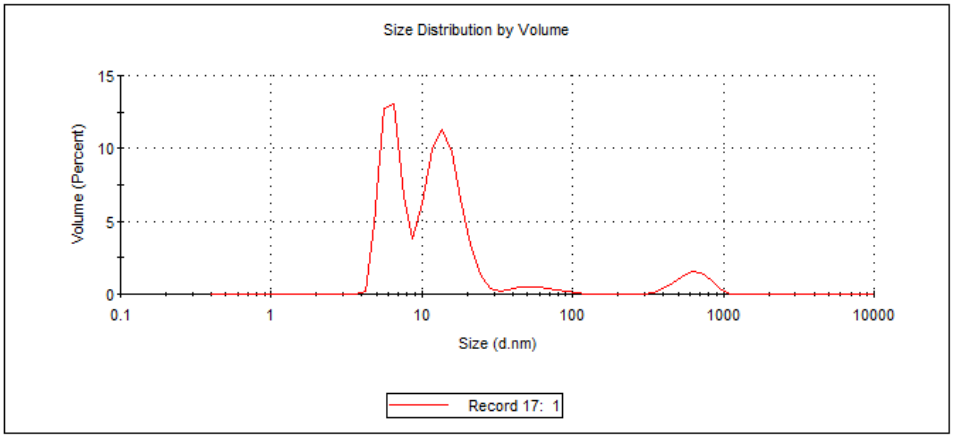
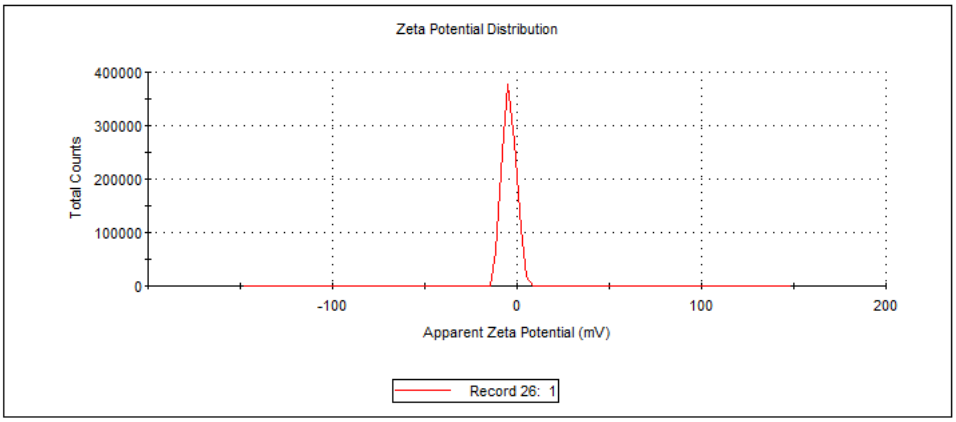


Figure 4 Zeta Potential of K10

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -4,25	Peak 1:	-4,25	100,0	3,71
Zeta Deviation (mV): 3,71	Peak 2:	0,00	0,0	0,00
Conductivity (mS/cm): 0,0996	Peak 3:	0,00	0,0	0,00

Result quality : Good



The particle size distribution is the widest of all the samples. In fact it has three peaks, this means that the copper nanoparticles have three different sizes; 51% of them have 14 nm; 6,5% have 637 nm and the rest, 2,7% have 58 nm. The

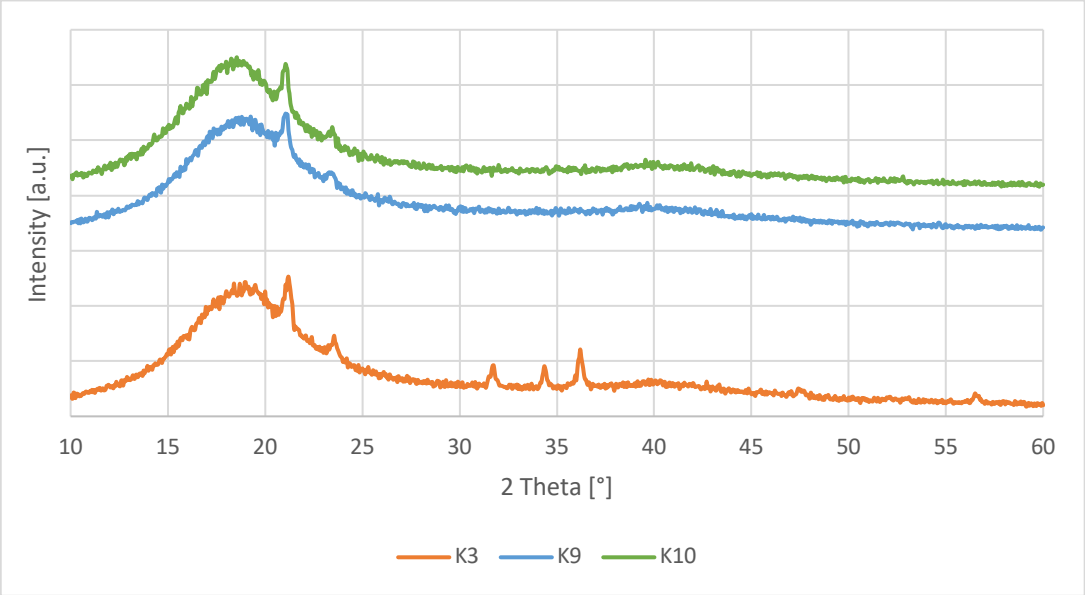
Zeta Potential plot shows that the peak is reached at -4,25 mV, which is not an acceptable value. The desired value should be around -20 mV. The conclusion is the same as in the other two samples, the suspension is not stable. Once we have obtained all the DLS results, it is time to perform the XRD analysis.

The X-Ray Diffraction (XRD) is a non-destructive test method used to analyze the structure of crystalline materials. Specifically it is used to identify the crystalline phases present in a sample. This test method is performed by directing an x-ray beam at a sample and measuring the scattered intensity as a function of the outgoing direction [6].

For this study, it is necessary to place a little amount each of sample in a Petri dish and let it dry for a day. Once it is dried, a thin layer is formed. This layer should be separated from the Petri and cut into a rectangle shape of approximately 2x1 cm. The apparatus used for XRD analysis is a X-ray diffractometer, named X'pert PW 1752/00, from Phillips Company.

Performing the XRD analysis of each sample, three curves are obtained (Figure 5).

Figure 5 XRD plot of K3, K9 and K10



From this XRD studies the main conclusion that can be obtained is, in the case of K3, there are 5 peaks (21, 24, 32, 35 and 36), which proves that there is ZnO in this sample. In K9 and K10, there are two peaks around 19 and 40, this justify the presence of organic matter in this samples (chitosan and gelatin).

The last study is Electron Microscopy / Energy Dispersive X-Ray Spectroscopy (SEM-EDS) analysis. A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample [8]. Energy Dispersive X-Ray Spectroscopy (EDS) is a chemical microanalysis technique used in conjunction with Scanning Electron Microscopy (SEM). The EDS technique detects x-rays emitted from the sample during bombardment by an electron beam to characterize the elemental composition of the analyzed volume [9]. For this study, it is necessary to introduce a very small solid sample of each sample in a container. This should be send to Copernicus University, in order for them to analyze it

and give us the relevant results. The apparatus used for SEM-EDS analysis is LEO Electron Microscopy Ltd, model 1430 VP.

Figure 6 Map of SEM-EDS results from K3

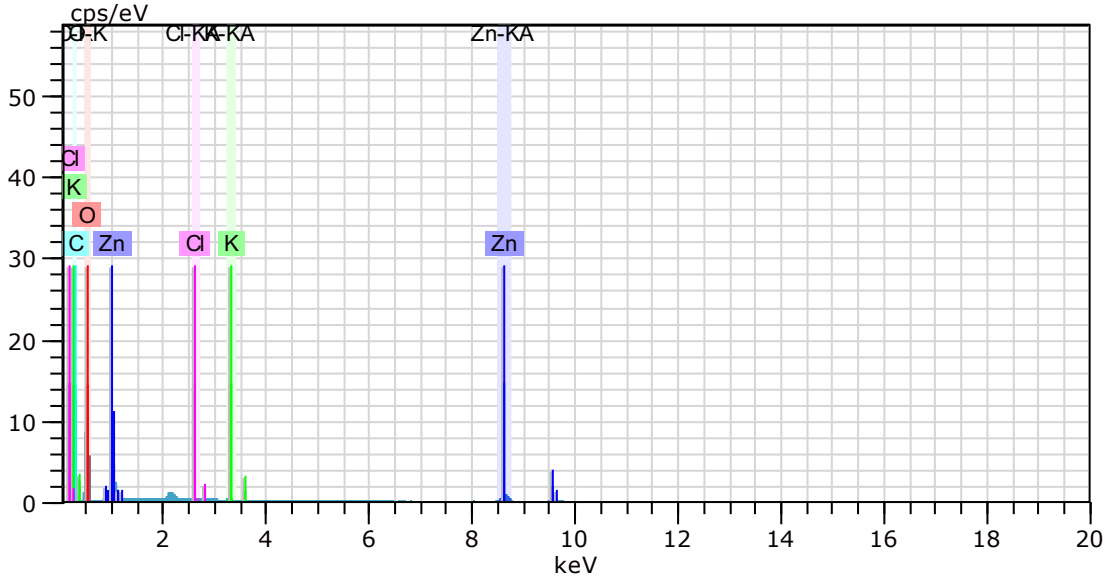


Figure 6.1 SEM-EDS results from K3

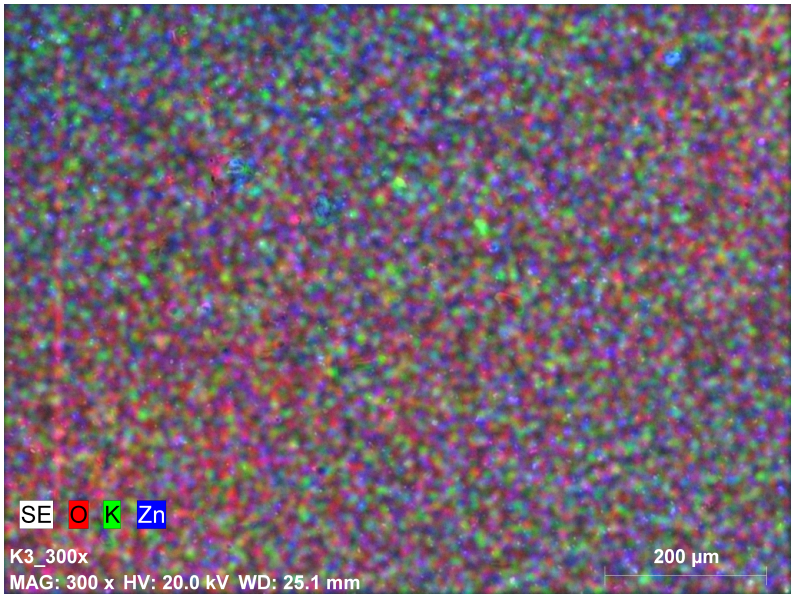


Figure 6 shows the components included in K3 and their respective amounts. The components are Cl, K, C, O, and Zn. By having this data we can justify the presence of ZnO nanoparticles in our sample. Figure 6.1 shows how the particles are placed in the sample, There are two big blue spot which correspond to ZnO nanoparticles. Moreover , the presence of K or Cl is due to the material of analysis. The presence of C is because there is organic matter in our sample.

Figure 7 Map of SEM-EDS results from K9

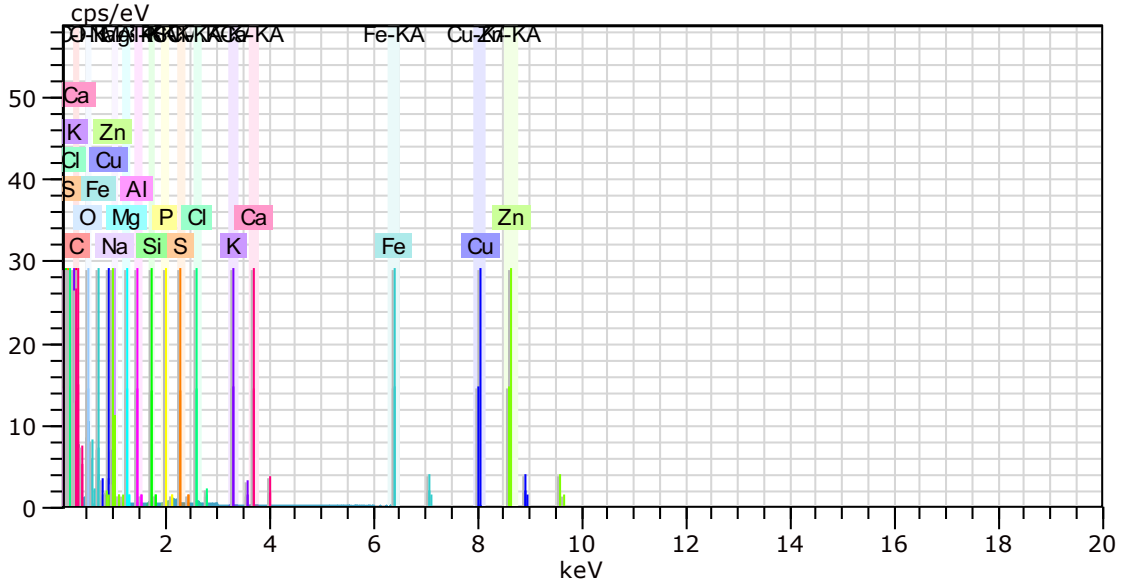


Figure 7.1 SEM-EDS results from K9

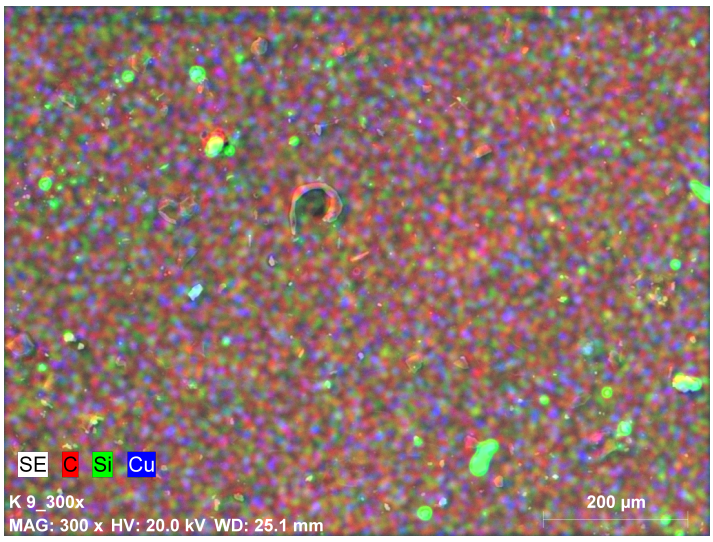
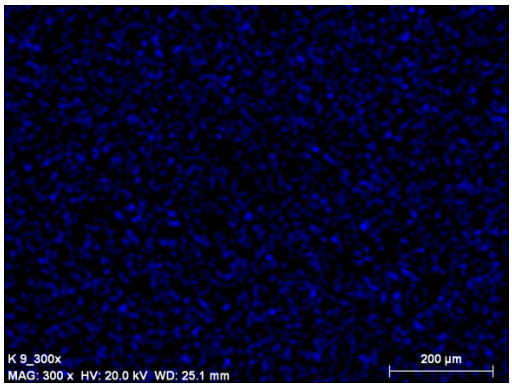


Figure 7 shows the components included in K9 and their respective amounts. The components are Si, K, Ca, S, Fe, Al, Mg, Na, Cl, Zn, C, O, and Cu. Also, it shows that the presence of Cu and Zn are dominating the sample above the rest. By having this data we can justify the presence of Cu nanoparticles in our sample. Figure 7.1 shows how the particles are placed in the sample. Moreover the presence of the rest of the compounds could be due to the material of analysis, because of the presence of remnants of samples or previous tests. The presence of C is because there is organic matter in our sample.

Figure 8 SE of Copper (Cu)



Analyzing Cu nanoparticles size, in order to ensure that DLS results were right, it is obtained that the particle size is around 4,4 µm (4400 nm). For the calculation of this parameter, one of the particles in the figure is transferred to the horizontal bar and this bar is separated into segments as small as the particle size. They are obtained 45 segments and just by dividing 200 µm among 45 segments, it is obtained that each particle has 4,4 µm, which are 4400 nm. This value is more or less the same as the obtained in DLS studies, so it can be concluded that this is the actual Copper nanoparticle size.

Figure 9 Map of SEM-EDS results from K10

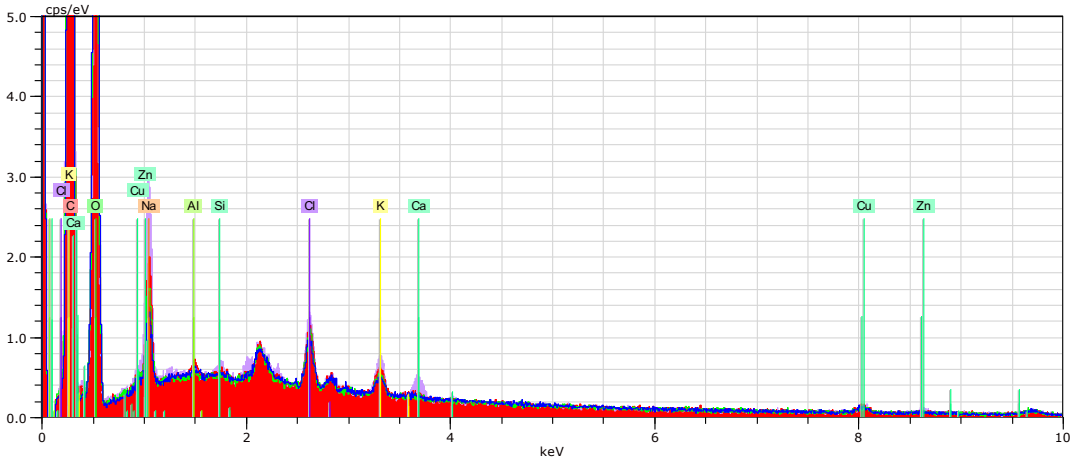


Figure 9.1 SEM-EDS results from K10

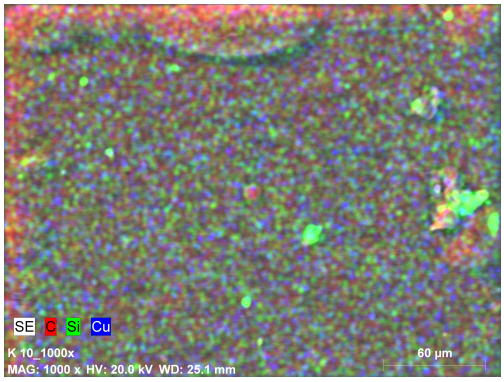


Figure 9 shows the components included in K10 and their respective amounts. The components are Si, K, Ca, Al, Na, Cl, Zn, C, O, and Cu. Also, it shows that the presence of Cu and Zn are dominating the sample above the rest. By having this data we can justify the presence of Cu nanoparticles in our sample. Figure 9.1 shows how the particles are placed in the sample. Moreover the presence of the rest of the compounds could be due to the material of analysis, because of the presence of remnants of samples or previous tests. The presence of C is because there is organic matter in our sample.

4. Antibacterial activity

With the introduction of nanotechnology, metal nanoparticles have gaining increased attention as antimicrobial agents due to their broad inhibitory spectrum against bacteria, fungi, and viruses. Some metal nanoparticles have received increasing interest as antimicrobials such as zinc oxide nanoparticles, since their antibacterial effects have been described. Metal nanoparticles can exert their effect on microbial cells by generating membrane damage, oxidative stress, and injury to proteins and DNA. Zinc oxide nanoparticles exhibit attractive antibacterial properties due to increased specific surface area as the reduced particle size leading to enhanced particle surface reactivity. ZnO is a bio-safe material that possesses photo-oxidizing and photocatalysis impacts on chemical and biological species. Interestingly, ZnO NPs are reported by several studies as non-toxic to human cells, this aspect necessitated their usage as antibacterial agents, noxious to microorganisms, and hold good biocompatibility to human cells. In the subsequent sections, we have discussed the factors affecting the antibacterial activity, including ZnO nanoparticle size and concentration[12]. In addition, Copper is a readily available metal and one of the essential elements in most living organisms. This metal has been also used as potential antimicrobial agent since ancient times. Copper ions have demonstrated antimicrobial activity against a wide range of microorganisms, such as Staphylococcus aureus, Salmonella enteric, Escherichia coli, etc. This material kills 99.9% of most pathogens within 2 h contact. Also, in some cases, this metal possesses properties better relative to the other expensive metals with antimicrobial activity, such as, silver and gold.

For instance, the Cu nanoparticles indicated higher antibacterial effect relative to the silver nanoparticles against E. coli. The copper surfaces can be used to kill bacteria, yeasts, and viruses which are known as "contact killing" (contact-mediated killing). Contact killing by copper was reported to occur at a rate no less than seven to eight logs per hour, and in general, subsequent to the extended incubation. No live microorganisms were recovered from the copper surfaces. This leads to the idea of using copper as a self-sanitizing material [11]. The microbiological studies are performed according to Polish Standard PN-EN 1650. The first step in these studies is to prepare series of dilutions of the samples (20 ml of each sample) and dilute them with water so as the final concentration is as shown in Table 7.

Table 7 Data for preparation of dilutions.

STARTING PRODUCT	INITIAL CONCENTRATION (ZNO/ CU)	DILUTIONS
K3	5%	1%; 2%; 3%, 4%
K9	200 ppm	12,5 ppm; 25 ppm; 50 ppm; 100 ppm, 200 ppm
K10	200 ppm	

This solutions should be prepared in 10 grams. For preparing this dilutions there are some calculations needed;

K3

4 % 4 g ------ 100 g
X ------ 10 g X= 0,4 g

5 g ------ 100 g
0,4 g ------ X

X= 8 grams of initial concentration
+ 2 grams of water

3 % 3 g ------ 100 g
X ------ 10 g X= 0,3 g

5 g ------ 100 g
0,3 g ------ X

X= 6 grams of initial concentration
+ 4 grams of water

2 % 2 g ------ 100 g
X ------ 10 g X= 0,2 g

5 g ------ 100 g
0,2 g ------ X

X= 4 grams of initial concentration
+ 6 grams of water

1% 4 g ------ 100 g
X ------ 10 g X= 0,1 g

5 g ------ 100 g
0,1 g ------ X

X= 2 grams of initial concentration
+ 8 grams of water

For the calculations of K9 and K10, it is important to take in account that the results are the same for both;

K9=K10

200 % 200 g ------ 1000 g
X ------ 10 g X= 2 g

200 g ------ 1000 g
2 g ------ X

X= 10 grams of initial concentration
+ 0 grams of water

100 % 100 g ------ 1000 g
X ------ 10 g X= 1 g

200 g ------ 1000 g
1 g ------ X

X= 5 grams of initial concentration
+ 5 grams of water

50 % 50 g ------ 1000 g
X ------ 10 g X= 0,5 g

200 g ------ 1000 g
0,5 g ------ X

X= 2,5 grams of initial concentration
+ 7,5 grams of water

25 % 25 g ------ 1000 g
X ------ 10 g X= 0,25 g

200 g ------ 1000 g
0,25 g ------ X

X= 1,25 grams of initial concentration
+ 8,75 grams of water

12,5 % 12,5 g ------ 1000 g
X ------ 10 g X= 0,125 g

200 g ------ 1000 g
0,125 g ------ X

X= 0,625 grams of initial concentration
+ 9,375 grams of water

Once we have made all the calculations, the next step is to autoclave the glass tubes by pouring 10 ml of water into them, cover each of them with aluminum film and put them into one big beaker (one that fits the autoclave chamber) and run autoclaving. After autoclaving, pour out

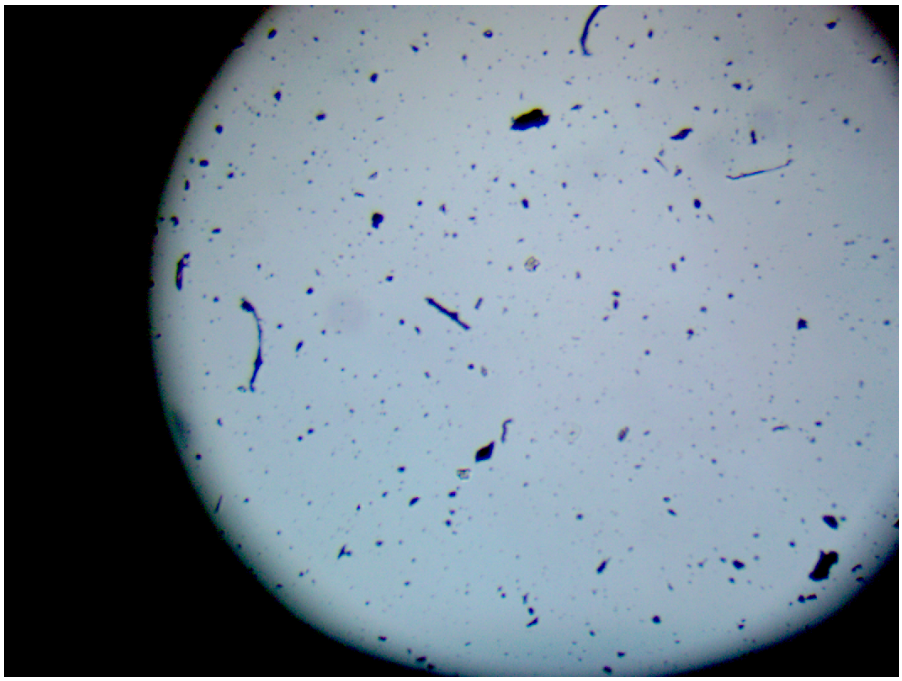
the water and keep them in the laminar chamber.

The following step is preparing tubes with neutralizing agent. For the preparation of the neutralizing agent Dey-Engley the first thing to do is to weight 19,5 grams of Dey-Engley compound and make up to 500 ml and once again autoclave it. Once we have the neutralizing agent prepared, we have to put 8 ml of it and 1 ml of water to autoclaves glass tubes. Then we have to keep them in the laminar chamber. The next step is to prepare Petri dishes with growth medium. The bacteria tested in this experiment will be Aspergillus niger, so it is necessary YPD medium. For the preparation of the solution of YPD medium, it is weight 8 grams of pepton, 4 grams of yeast extract, 8 grams of sucrose and 8 grams of agar. This is mixed with water and make up to 400 ml in a bottle of 500 ml and autoclave the bottle. This bottle should be kept in the laminar chamber. Also it is necessary to pour 12 ml of liquid MEA into Petri dishes and leave them for solidification.

In this step there have been a problem with the solidification of YPD medium, so it was substituted for Escherichia Coli. In order to ensure that the medium of Escherichia Coli is in the appropriate concentration range, it is necessary to take a drop of the medium on Fuchs-Rosenthal chamber and check the amount of cells, using optical microscope (Figure x).

The concentration should be between $1{,}5\times10^7$ cfu/ml – $5{,}0\times10^7$ cfu/ml. If the amount is greater, the suspension must be diluted.

Figure 10 Echerichia Coli in the optical microscope



In order to calculate the concentration of Escherichia Coli, this formula is applied;

$$[\,]\ of\ E.Coli\ = \frac{cells\ in\ square\ x\ dilution\ x\ 80}{number\ of\ squares}$$

So the first step is to determine the number of cells in a square by assessing a square and counting the cells. Doing this is obtained that there are 160 cells in a square. In addition, it is known that dilution is 1, so it is possible to determine the concentration of E. Coli;

$$[\,]\ of\ E.Coli\ = \frac{160\ x\ 1\ x\ 80}{1} = 12800\frac{c.f.u}{mm^3}$$

Where c.f.u stands for colony forming unit. The value of 80 comes from the own Fuchs-Rosental chamber device; the surface (0,0625 mm^2) is multiplied by the height (0,2 mm) and it is obtained 0,0125 m^3. So the inverse of that is 80 $[m^3]^{-1}$. Changing the units into ml, it is obtained that the concentration of E. Coli is $1{,}28 \cdot 10^7$ c.f.u/ml. This value is more or less in the range of concentrations, assuming that it could be an error in the counting of the cells. So it can be concluded that the medium is appropriate and continue with the procedure.

Once the medium has solidified after some days, the next step is to start with the procedure itself; in the laminar chamber, in order to autoclave glass tube, it is poured 4 ml of tested material, 0,5 ml of water and 0,5 ml of tested suspension of microorganism. This tube has to be shaken and left for specific time. There are four different time for each sample; 5, 10, 30 and 60 minutes. After this specific time, 1 ml of suspension should be taken and introduced to previously prepared tube with neutralizing agent and with water. It has to be shaken variously, in order to get the tubes well neutralizing. It takes around 5 minutes in each case. After this time, it is necessary to take 1 ml of suspension an put it on previously prepared Petri dishes with solidified medium. These dishes should be incubated in 30ºC for 24, 48 and 72 h. After each time, it is necessary to take pictures of the cultivations for the correct assessing of the share of cultivated dish in relation to the whole dish. As mentioned, the next step is to assess the best concentration for the disappearing of the microorganisms and how nanoparticles have eliminated these microorganisms through the days. The comparison will be done by taking different photos before 24, 48 and 72 hours of the front and back side of the Petri dishes.

Figure 11 Petri dishes of K3 after 5 min in contact with E. Coli

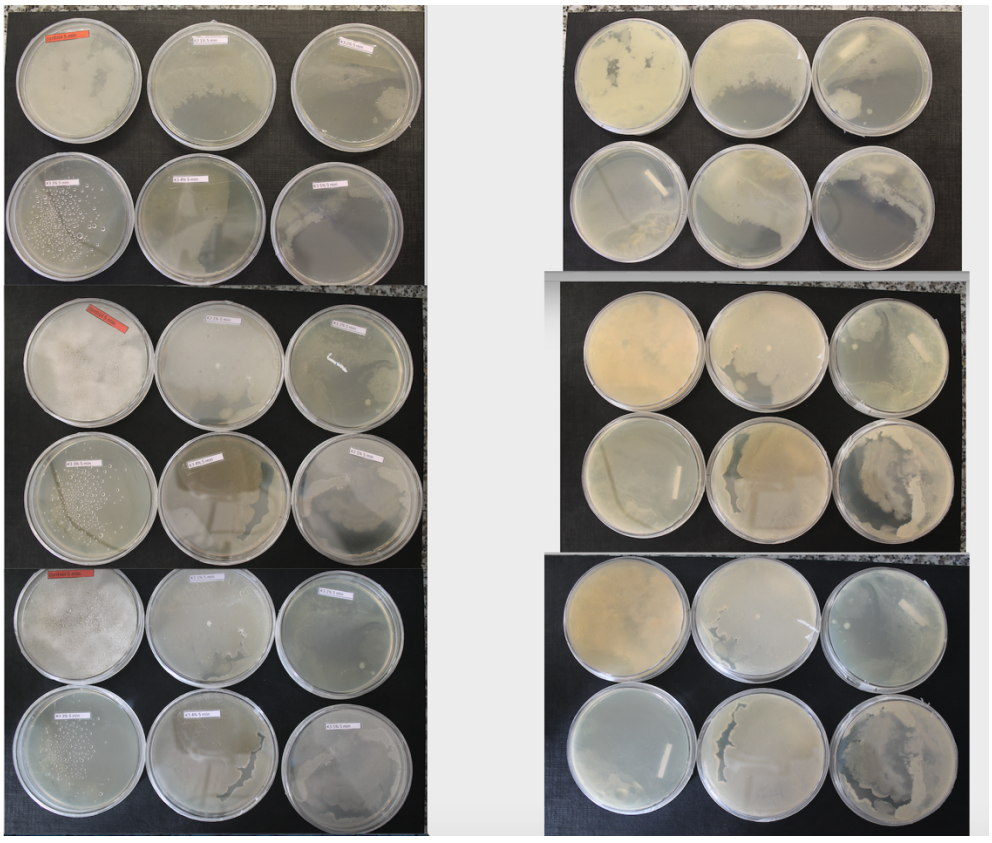
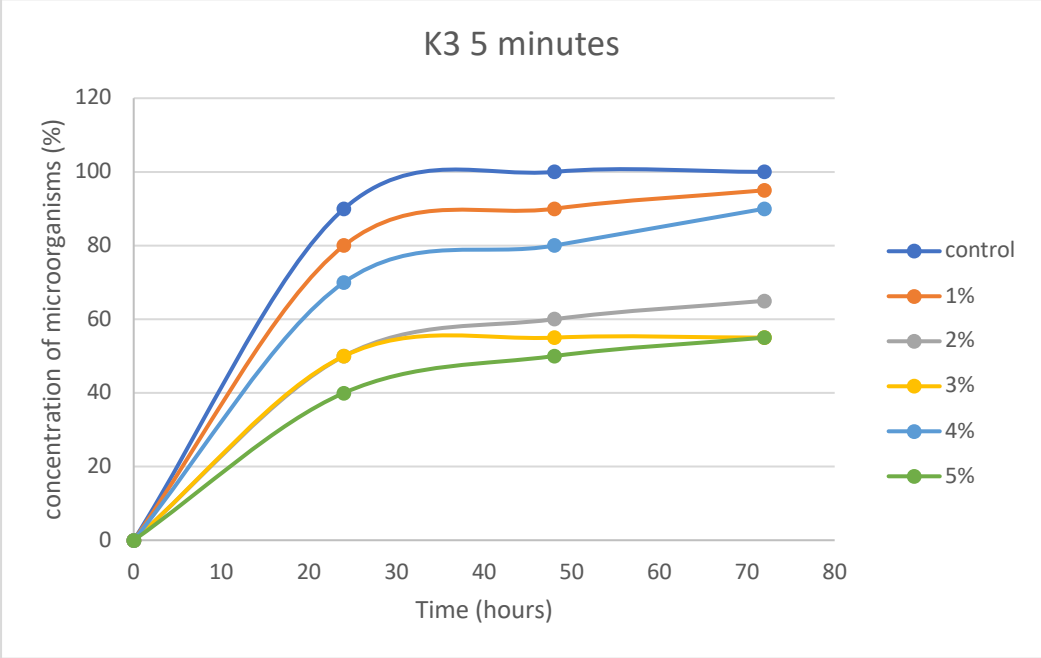


Figure 11. 1 Graphic of concentration of microorganisms (%) with time



This Figure 11 shows Petri dishes (front and back side) of K3 after 5 minutes in contact with E. Coli. The first row belongs to the photos taken 24 hours after the first contact, the second row belongs to 48 hours after the first contact and the last row belongs to 72 hours after the first contact. The Petri next to the one with the red label (up in the middle) corresponds to the lowest concentration of ZnO (1%), while the last Petri (the one in the bottom right) corresponds to the highest concentration of ZnO (5%).

The Petri dish with the red label is the reference sample (it only has H_2O), so it seems clear that it would be the sample with the greatest growth of microorganisms. The photos show exactly this behavior in this Petri, a great and rapid growth of organisms and in comparison with the other Petri, the growth rate is considerably higher. It is seen that the Petri with the lowest concentration has the highest rate of growth. This seems logical. However, the Petri with the highest concentration (5%) of ZnO nanoparticles does not correspond with the Petri with the lowest growth of microorganisms in this case. In fact, the best concentration of ZnO when K3 is putting in contact with E. Coli for 5 minutes, is 3%. It is seen that for 2% at 72 hours the microorganisms disappear totally, as in 3%. In the cases of 4% and 5% the inhibition of growth is low (the concentration of microorganisms is high).

Figure 12 Petri dishes of K3 after 15 min in contact with E.Coli

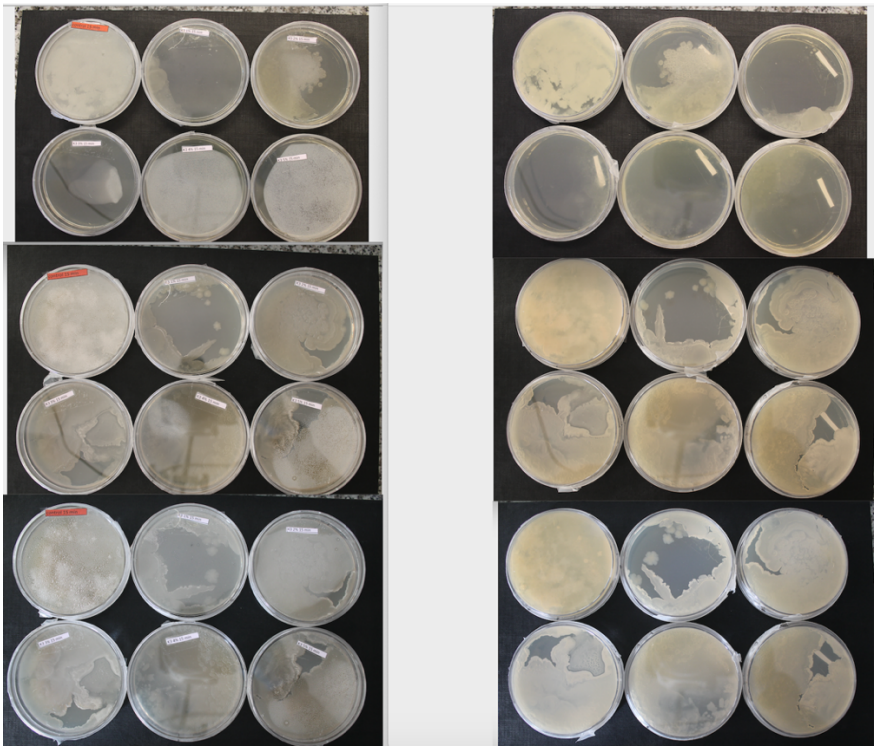
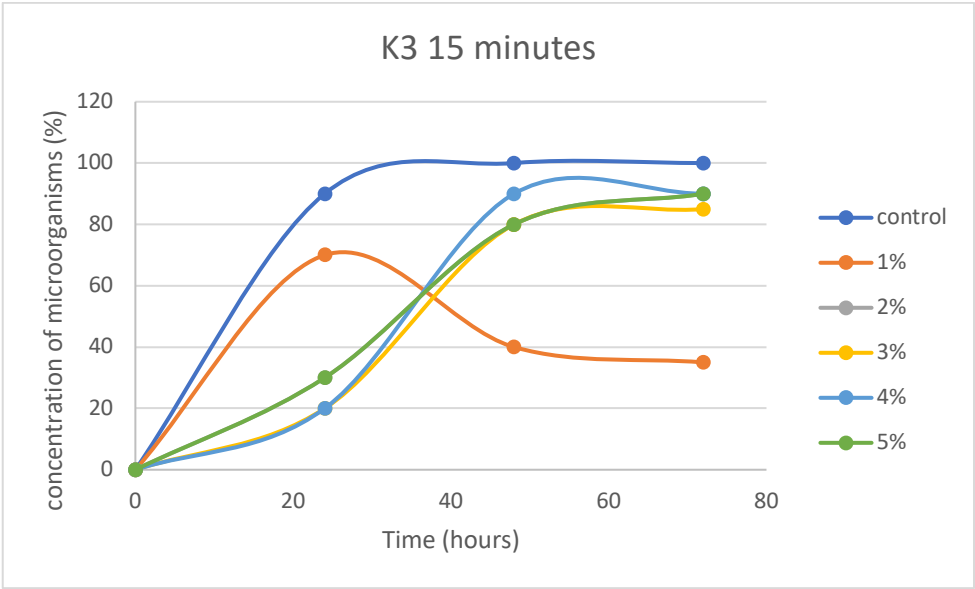


Figure 12.1 Graphic of concentration of microorganisms (%) with time



In this case it happens exactly the same with the red labeled Petri, it has the greatest growth of microorganisms due to its concentration (only H_2O). However, in this case happens something curious with the highest concentration (5%), since it has really high growth rate of microorganism (inhibition of growth is low). The same happens with the concentrations of 2, 3 and 4%, they have really small inhibition rate, so the microorganism growth velocity is considerably high. In this case, it happens just the opposite of what happened with

the previous example; the lowest concentration (1%) has the lowest growth rate, so that would be the best concentration when K3 is putting in contact with E. Coli for 15 minutes. It is important to highlight here that, although the inhibition rate in 1% concentration is not really good, it is the best in this case.

Figure 13 Petri dishes of K3 after 30 min in contact with E. Coli

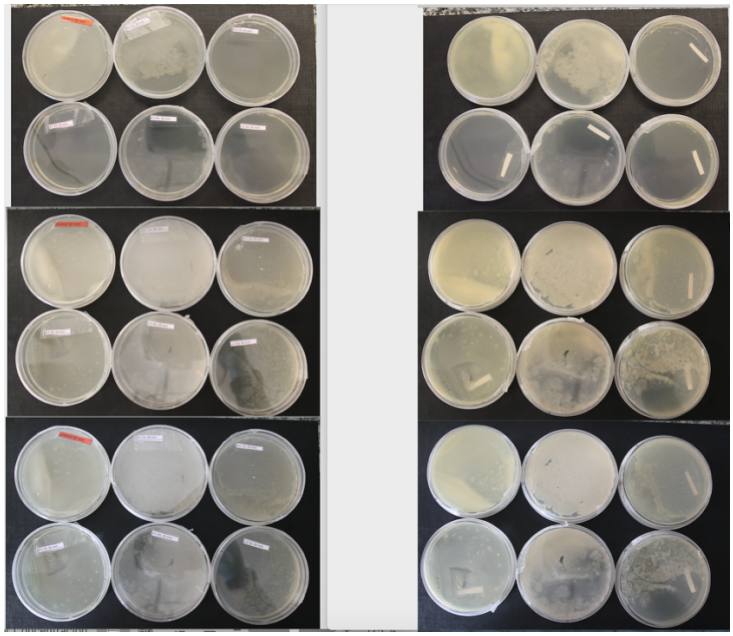
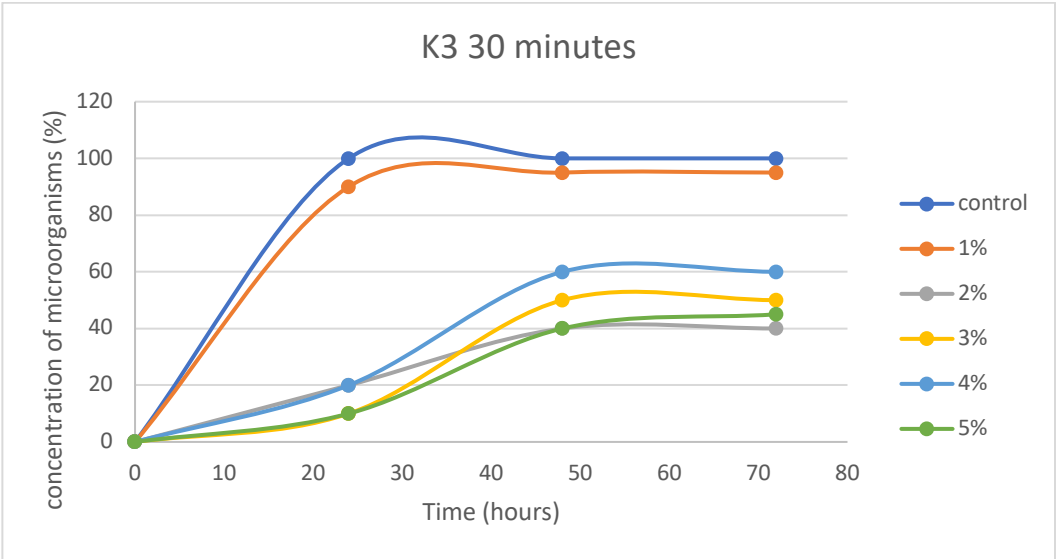


Figure 13.1 Graphic of concentration of microorganisms (%) with time



The same happens with the control Petri, it presents the greatest concentration of microorganism (the lowest inhibition rate). The Petris which have the lowest inhibition rate (except the control one) are from 1% and 4% concentrations of ZnO. On the other hand, the Petris with 2, 3 and 5% present a really good inhibition rate, due to the low microorganisms growth velocity. It is difficult to assess the best one between those three in this case, but 5% is the one who present the lowest

concentration of microorganism and the "clearest" Petri, so that would be the best concentration (5%) when K3 is putting in contact with E. Coli for 30 minutes. It is important to note here, that for the moment these Petris (K3 after 30 min in contact with E. Coli) are the ones who present the best growth inhibition rate.

Figure 14 Petri dishes of K3 after 60 min in contact with E.Coli

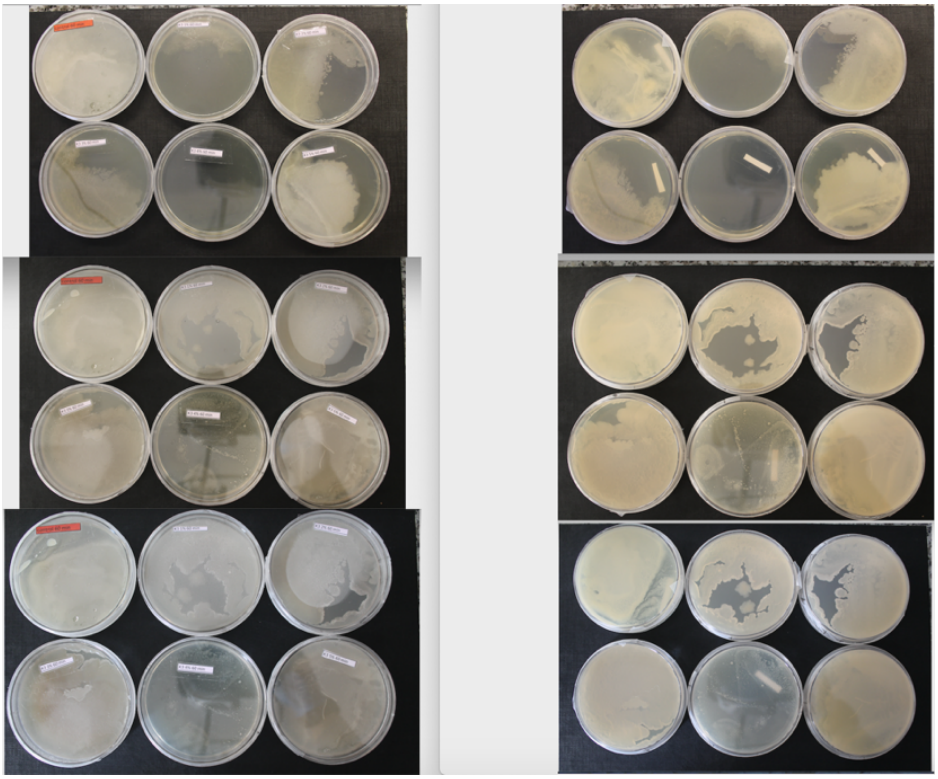
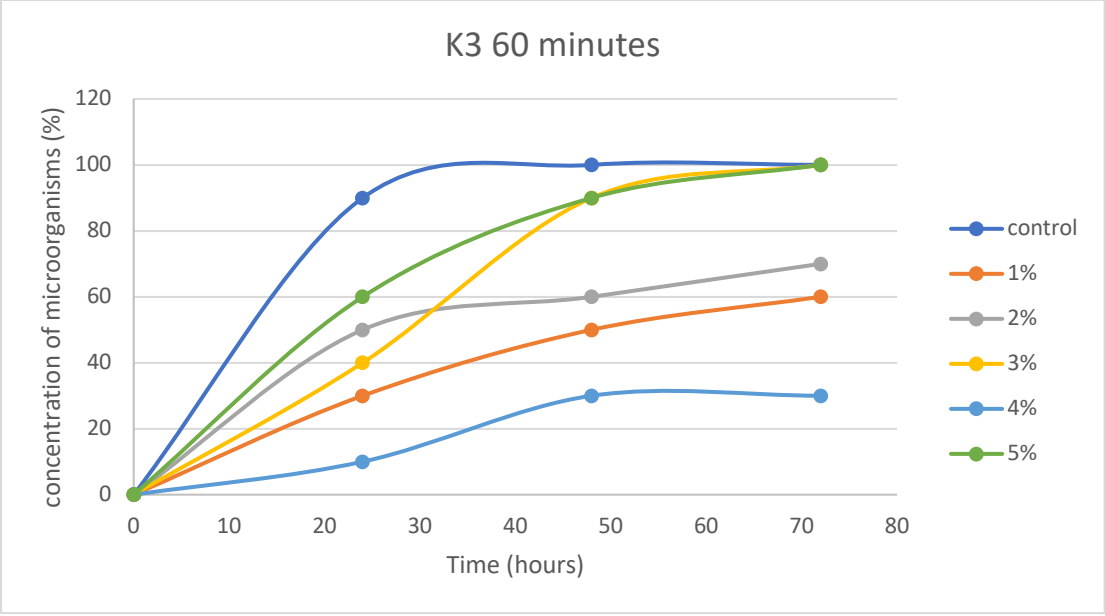


Figure 14.1 Graphic of concentration of microorganisms (%) with time



The control Petri, as in the other examples is the one with the lowest growth inhibition rate. In this case it is clear that 4% is the best concentration of ZnO, because is the only one who presents a reasonable inhibition growth rate, having the lowest concentration of microorganism. The other concentrations (1,2,3 and 5%) are a considerable high growth velocity of the microorganisms, which leads to a great concentration of microorganisms in each Petri. The worst concentration are 3 and 5%, which behave more or less as the control Petri.

Figure 15 Petri dishes of K9 after 5 min in contact with E.Coli

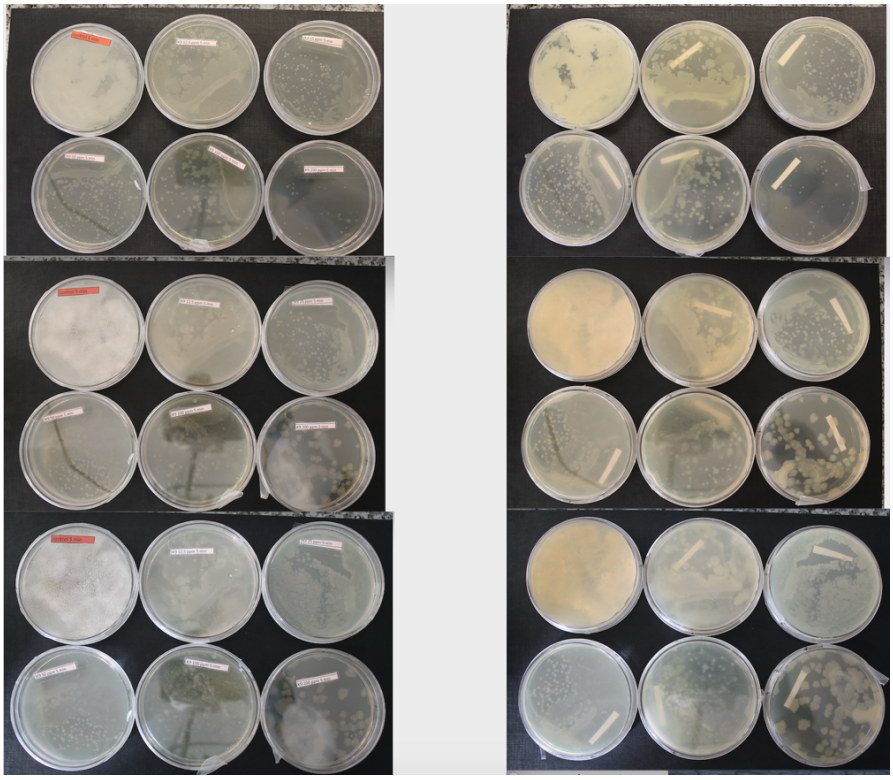
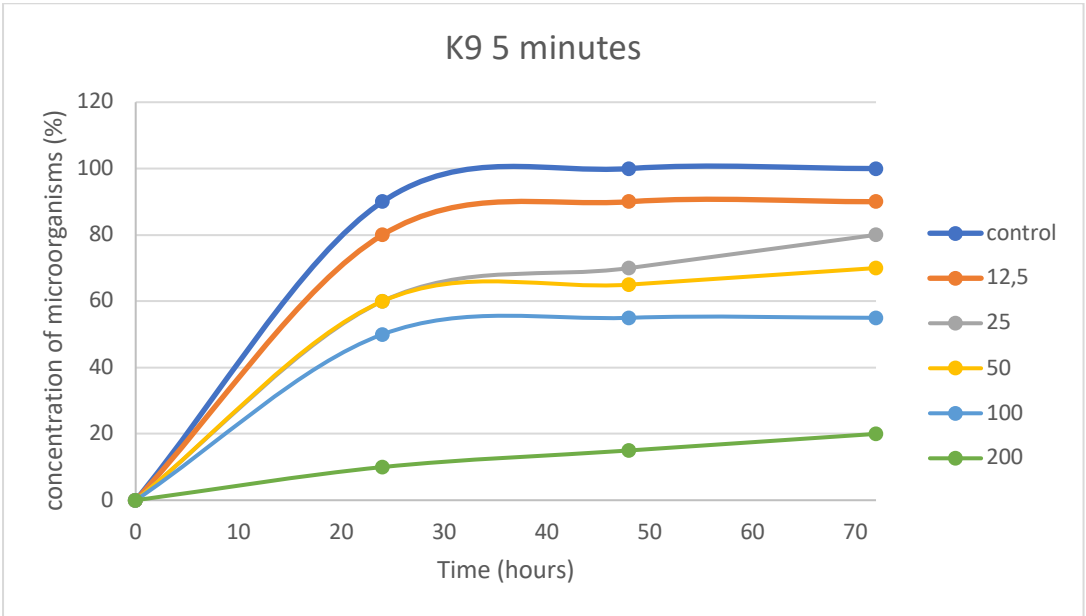


Figure 15.1. Graphic of concentration of microorganisms (%) with time



This figure shows Petri dishes (front and back side) of K9 after 5 minutes in contact with E. Coli. The first row belongs to the photos taken 24 hours after the first contact, the second row belongs to 48 hours after the first contact and the last row belongs to 72 hours after the first contact. The Petri next to the one with the red label (up in the middle) corresponds to the lowest concentration of Cu (12,5%), followed by 25, 50, 100 ppm and the last Petri (the one in the bottom right) corresponds to the highest concentration of Cu (200 ppm). The Petri dish with the red label is the reference sample (it only has H_2O), so it seems clear that it would be the sample with the greatest growth of microorganisms. It is clear in this figure that as the concentration of Cu nanoparticles increases, the growth inhibition too. That means that as the concentration increases, so does the antibacterial activity efficiency. Following this logic and just by looking at Figure 15, we can conclude that the best concentration (understanding it as the Petri with the lowest microorganism concentration) is 200 ppm, in other words, the Petri with the highest Cu nanoparticles concentration. In this case, the inhibition rate is considerably high, although 200 ppm is the best concentration, the others also have a remarkable inhibition rate.

Figure 16 Petri dishes of K9 after 15 min in contact with E.Coli

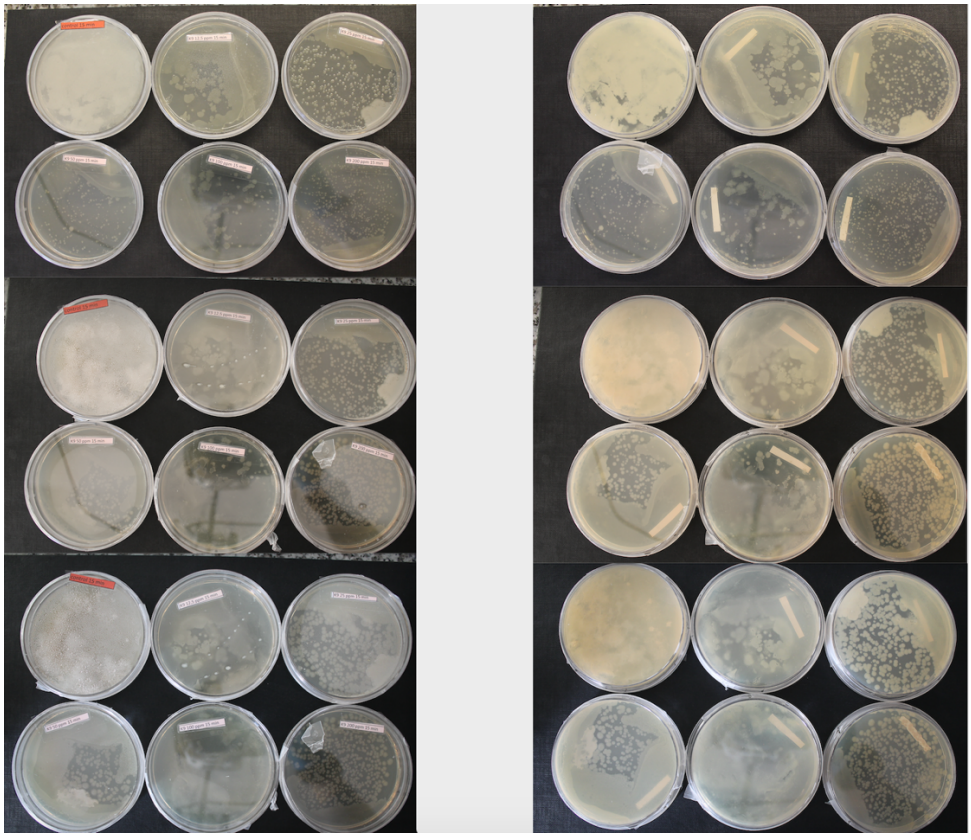
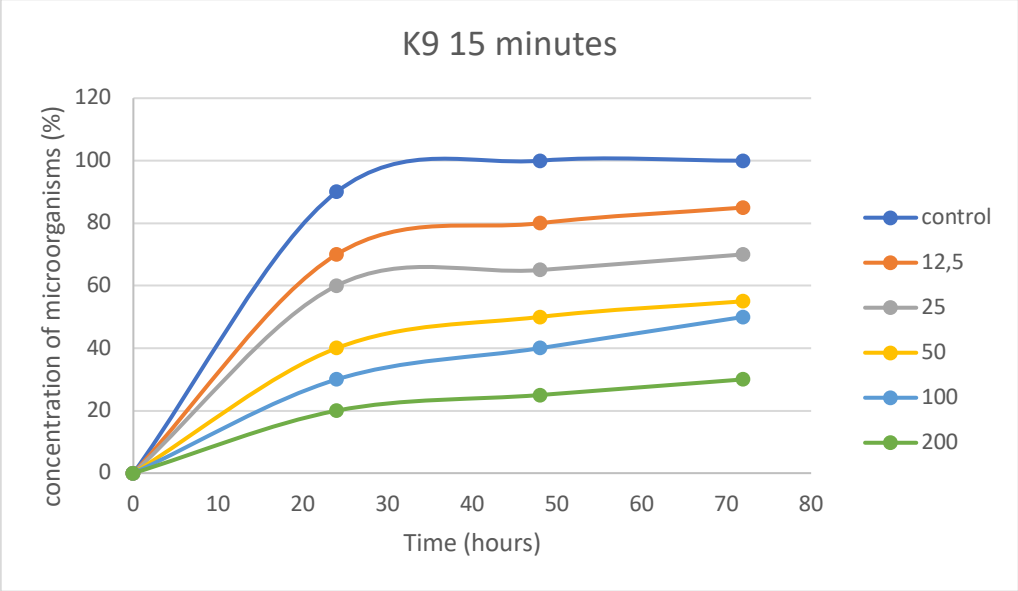


Figure 16.1. Graphic of concentration of microorganisms (%) with time



In this Figure 16 we can see that in general the inhibition is a little bit lower than in 5 minutes Petris. The control Petri has the greatest concentration of microorganisms. Once again the best concentration is the highest one (200 ppm), because it has the highest inhibition of growth rate. It is clear also in this figure that as the concentration of Cu nanoparticles increases, the growth inhibition too. That means that as the concentration increases, so does the antibacterial activity efficiency. In this case the inhibition rate is not as high as in the previous one, but is acceptable, remarking the 200 ppm concentration.

Figure 17 Petri dishes of K9 after 30 min in contact with E. Coli

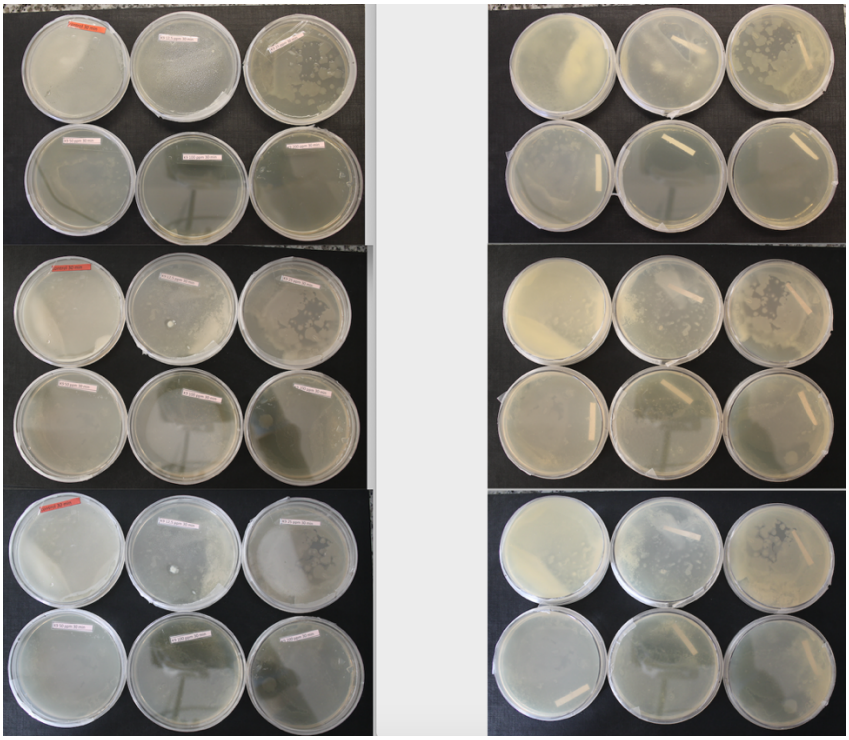
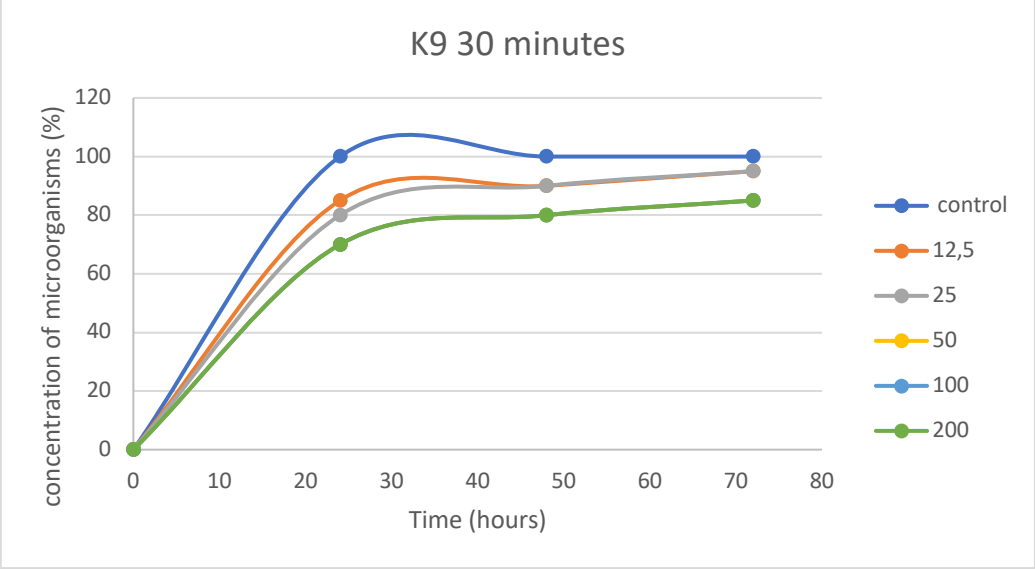


Figure 17.1. Graphic of concentration of microorganisms (%) with time



In this Figure 16 we can see that in general the inhibition is a little bit lower than in 5 minutes Petris. The control Petri has the greatest concentration of microorganisms. It happens something curious because the three highest concentration present the same behavior in this suspension at this specific time, they have more or less the same growth inhibition rate, so there is not just one best concentration. Although these three (50, 100 and 200 ppm) are the three best concentrations in this case because they present the lowest concentration of microorganisms, the inhibition is quite worse than in the other two cases. The worst concentration s are 12,5 and 25 ppm, which present a really low growth inhibition rate.

Figure 18 Petri dishes of K9 after 60 min in contact with E.Coli

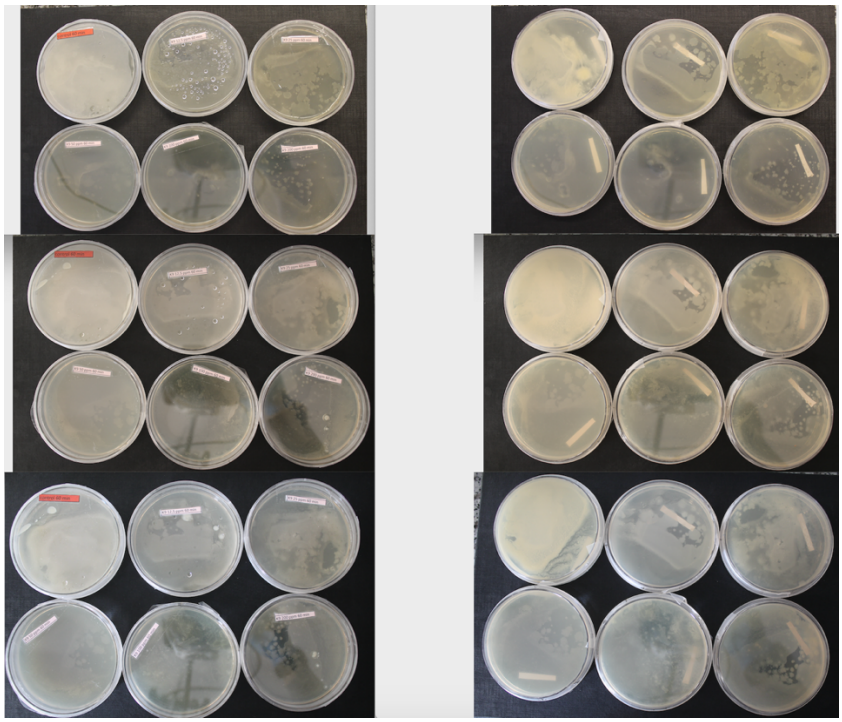
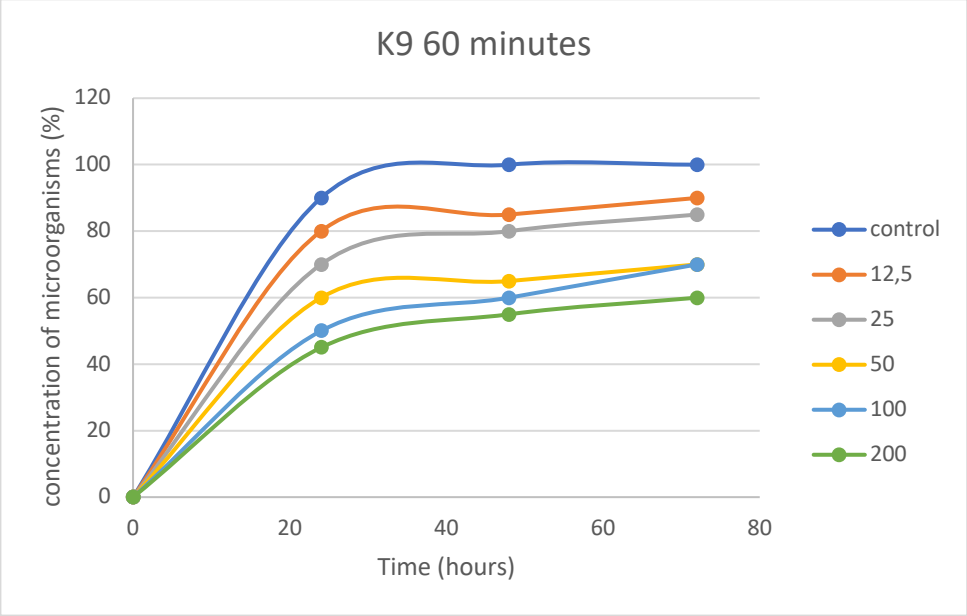


Figure 18.1. Graphic of concentration of microorganisms (%) with time



In this case the inhibition of growth is similar as the previous case. Is much worse than the first and second case. The control Petri has the greatest concentration of microorganisms. The Petri with the lowest concentration of microorganism is once again the one with highest concentration of Cu nanoparticles (200 ppm). But as said, the results obtained are not as good as in other cases. The worst concentration are 12,5 and 25 ppm, which present a really low growth inhibition rate. And finally, the analysis of K10.

Figure 19 Petri dishes of K10 after 5 min in contact with E.Coli

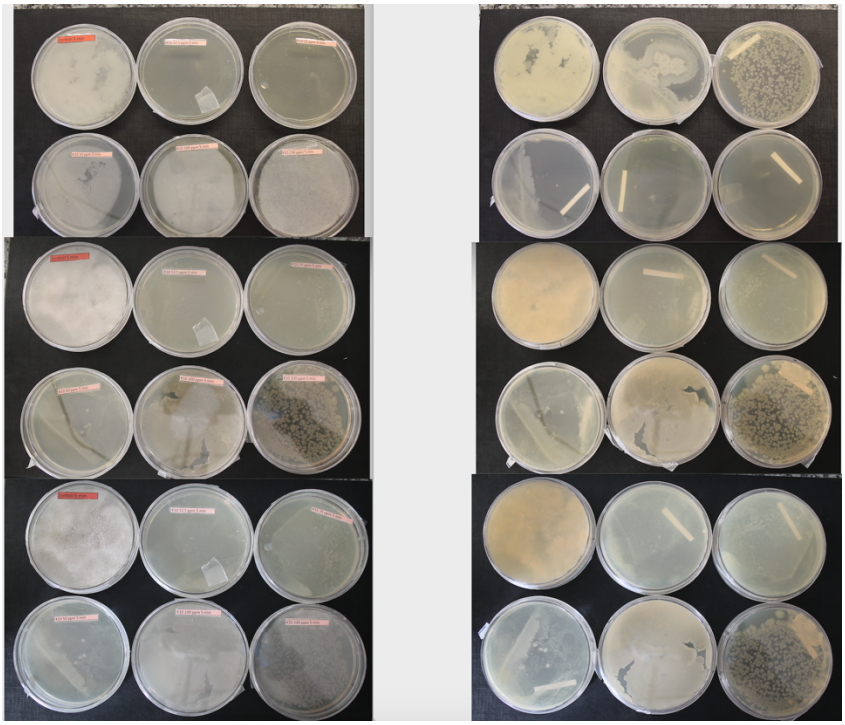
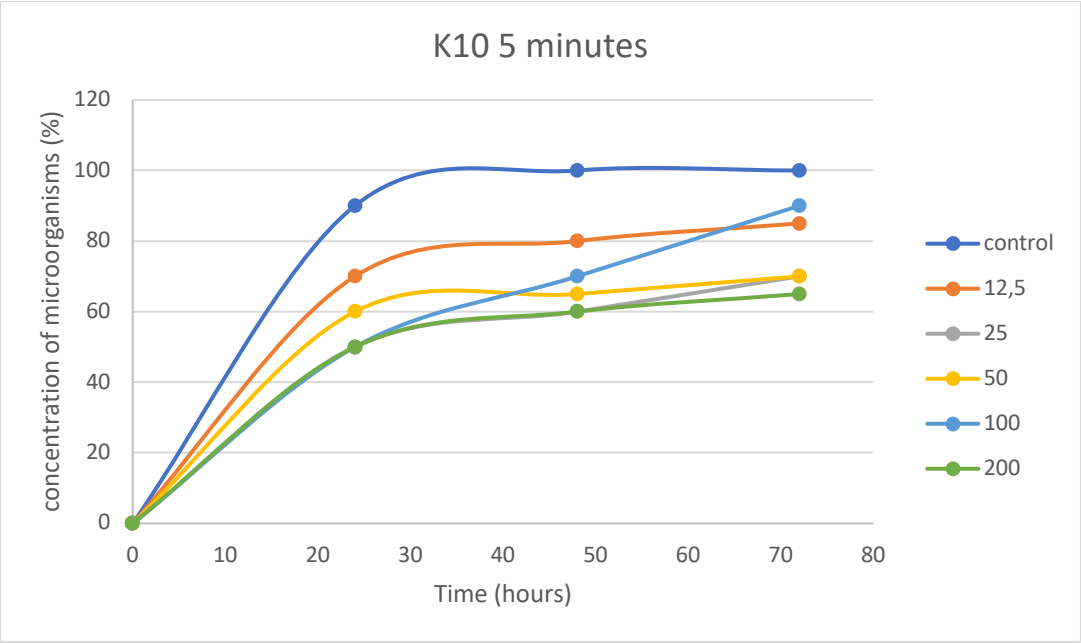


Figure 19.1. Graphic of concentration of microorganisms (%) with time



This figure shows Petri dishes (front and back side) of K9 after 5 minutes in contact with E. Coli. The first row belongs to the photos taken 24 hours after the first contact, the second row belongs to 48 hours after the first contact and the last row belongs to 72 hours after the first contact. The Petri next to the one with the red label (up in the middle) corresponds to the lowest concentration of Cu (12,5%), followed by 25, 50, 100 ppm and the last Petri (the one in the bottom right) corresponds to the highest concentration of Cu (200 ppm). The Petri dish with the red label is the reference sample (it only has H_2O), so it seems clear that it would be the sample with the greatest growth of microorganisms. In theory, results of K10 should be similar or practically the same as in K9, because the concentration of them is the same. Maybe there will be some changes, due to some small changes in the conditions of synthesis. On the other hand, it it seen plainly that the Petri with the greatest concentration of microorganisms is the one with 100 ppm. This is rare due to its high concentration of Cu nanoparticles, because as said before, as increasing the concentration of nanoparticles it should increase the efficiency of the antibacterial activity. In fact, it is true that the Petri with the lowest concentration of microorganisms and therefore the highest inhibition of growth is 200 ppm, which is the Petri with the highest concentration of Cu nanoparticles. In this case in general the growth inhibition rate is pretty low. This is shown more visually in 18.1 Figure, where it is seen that the best concentration is 200 ppm but it is not quite good, having a similar behavior as 50 and 25 ppm concentrations. In this case we can see that there is a big change in the behavior of the microorganisms; in general in this case the growth inhibition is much lower than in K9.

Figure 20 Petri dishes of K10 after 15 min in contact with E.Coli

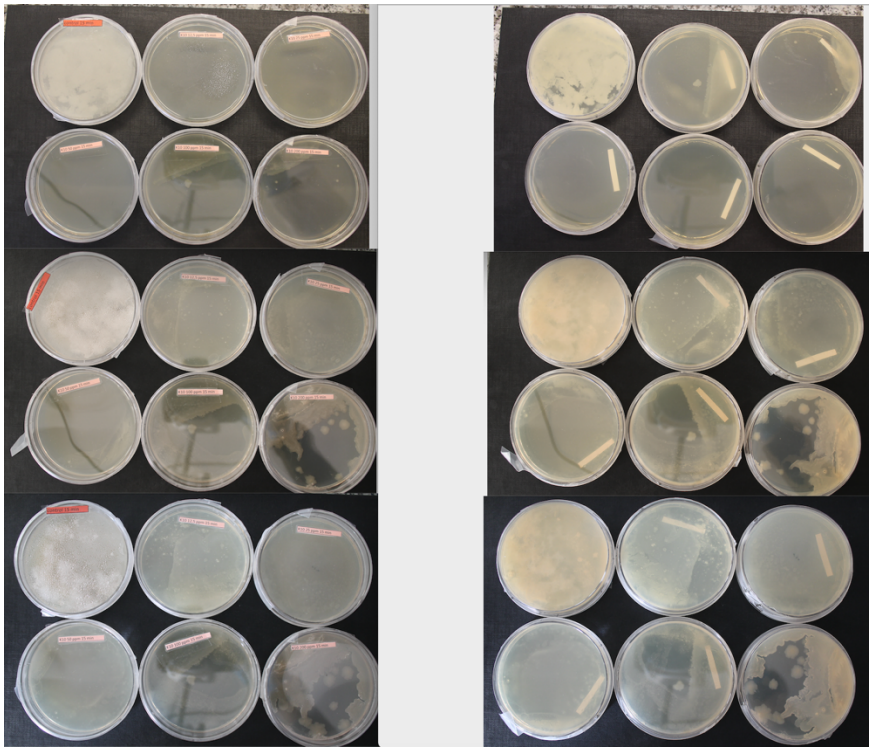
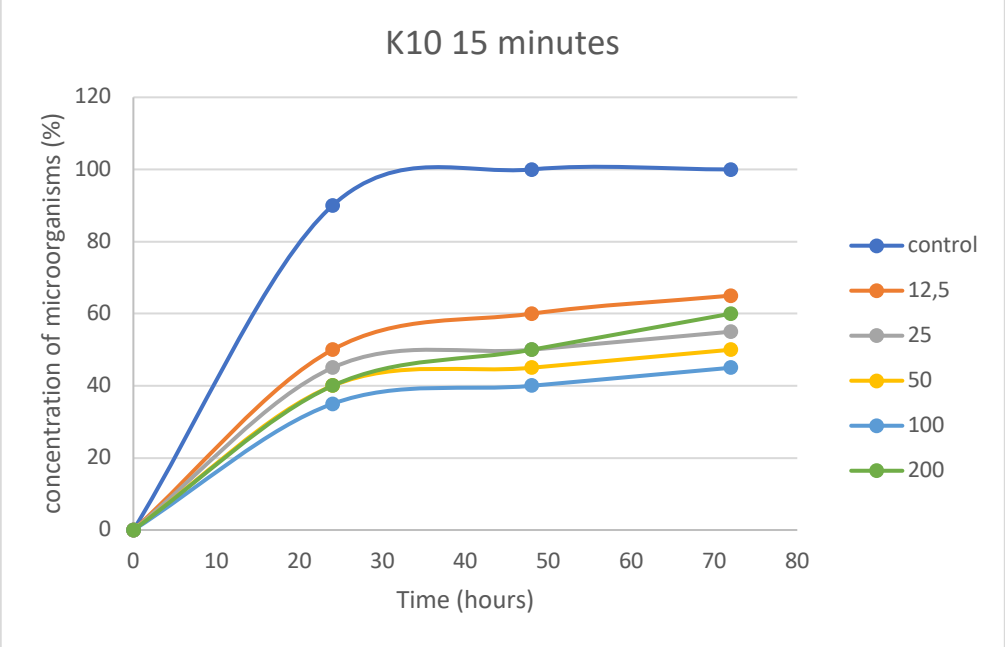


Figure 20.1. Graphic of concentration of microorganisms (%) with time



In this figure it happens exactly the opposite as in the previous one; the first thing that attracts attention is that the Petri with the greatest concentration of microorganisms is the one with the highest concentration of Cu nanoparticles (200 ppm), which means that this is the worst concentration in this case. This is rare because the efficiency of the antibacterial activity supposes to increase as the concentration of nanoparticles increase. On the other hand, it is seen that

the Petri with the lowest concentration of microorganism is, indeed, the one with 100 ppm of concentration, which is not the highest one but the second higher concentration of all. Comparing this with K9, once again the growth inhibition rate is pretty lower that in K9.

Figure 21 Petri dishes of K10 after 30 min in contact with E. Coli

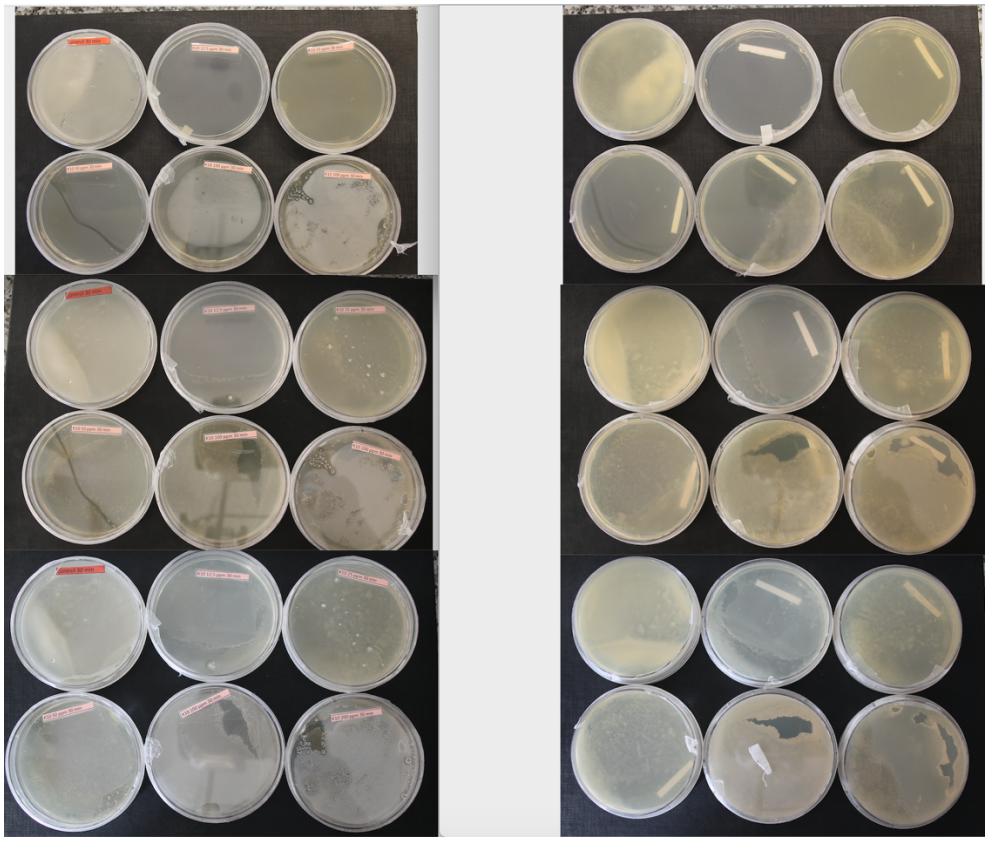
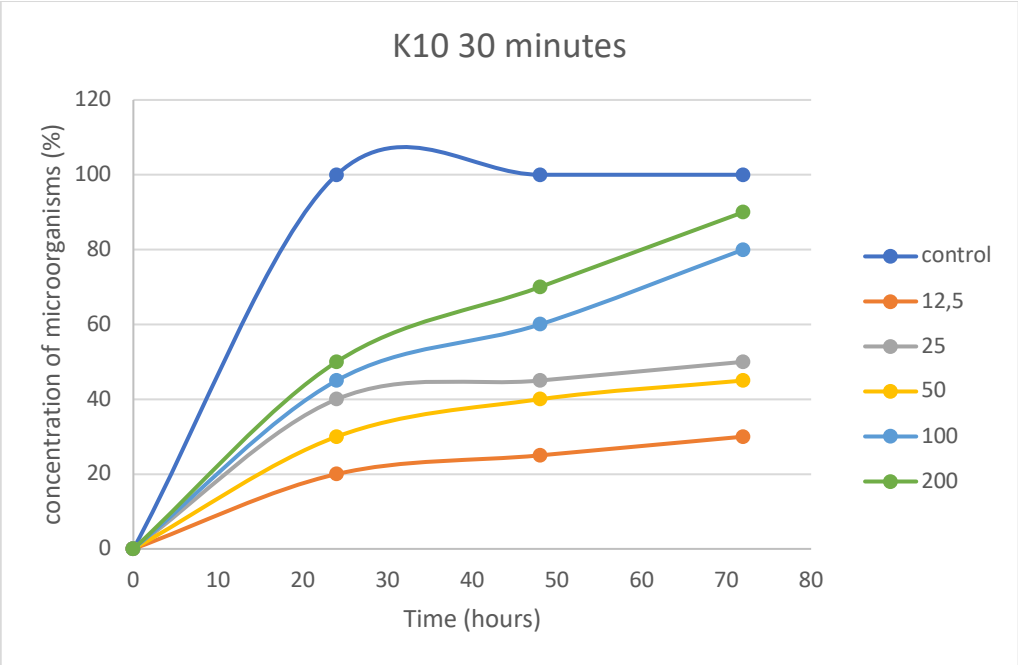


Figure 21.1. Graphic of concentration of microorganisms (%) with time



In this case it is seen rapidly by Figure 21.1 that the Petri with lowest concentration of microorganisms is the one which concentration is 12,5 ppm. This means that the lowest concentration of Cu nanoparticles leads to the lowest concentration of microorganisms. In addition, in this specific case the highest concentration of Cu nanoparticles gives place to the greatest concentration of microorganisms. As said before, in theory with the increasing of the concentration of nanoparticles, there should be a decreasing in the concentration of microorganisms. Therefore, this case does not match the theory. Once again it is seen that the growth inhibition rate falls considerably.

Figure 22 Petri dishes of K10 after 60 min in contact with E .Coli

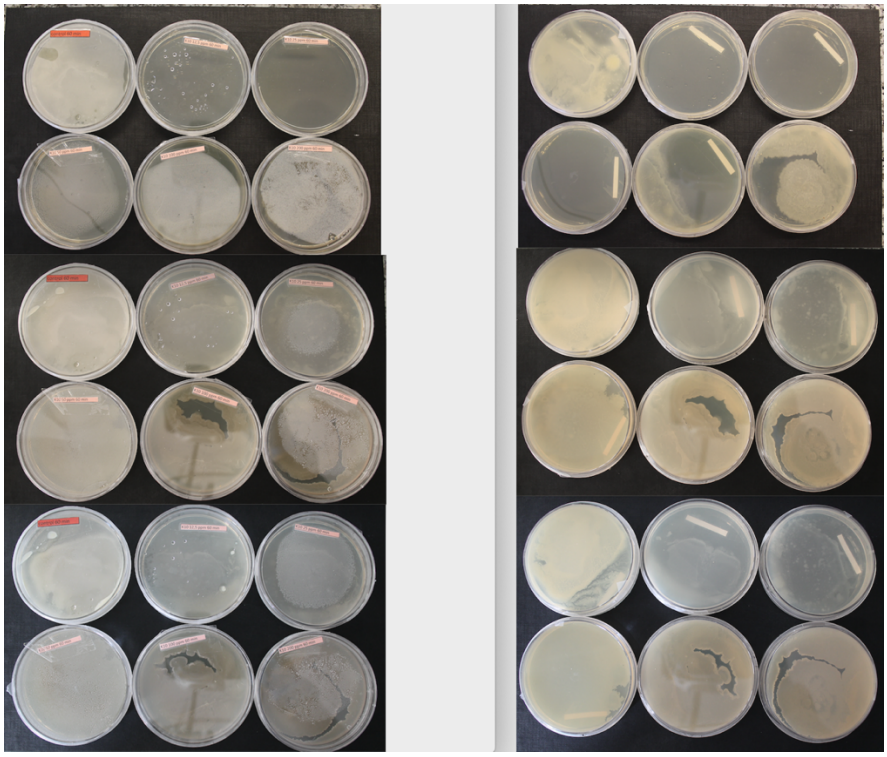
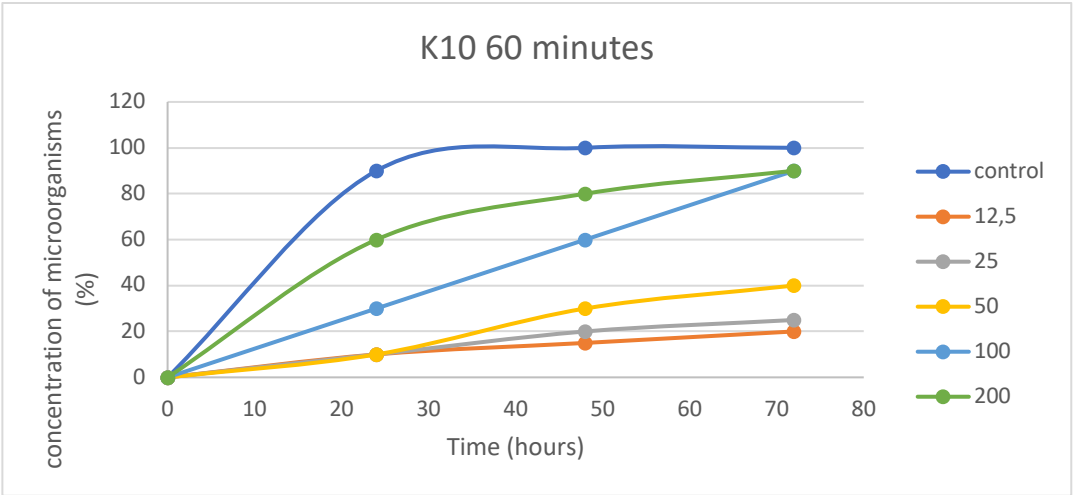


Figure 22.1. Graphic of concentration of microorganisms (%) with time



In this case occurs exactly the same as in the previous one; the Petri with lowest concentration of microorganisms is the one which concentration is 12,5 ppm. This means that the lowest concentration of Cu nanoparticles leads to the lowest concentration of microorganisms. In addition, in this specific case the highest concentration of Cu nanoparticles gives place to the greatest concentration of microorganisms. In fact, the two Petris with the highest concentration of nanoparticles are those with the highest concentration of microorganisms, too. In other words, the highest concentration in this case (and the previous one too), leads to a lower antibacterial activity.

In the next section the reasons of the different behavior of the samples will be discussed and with that the main conclusions can be extracted.

5. Discussion and conclusions

This section is going to relate the physico-chemical properties and antibacterial properties with the final efficiency of the nanoparticles and their behavior in presence of microorganisms.

Particle size play an important role in the efficiency of antimicrobial activity. This parameter is influenced by the pH. In fact, pH plays a key role in nucleation and growth of nanoparticles. In similar study, Zhang et al[13]reported that pH value is the main factor that influences the morphology of the nanoparticles. They considered that the nucleation profoundly depends on the pH of the starting solutions. Looking at this experiment, it is seen that K3 and K10 have more or less the same pH (around 6,7) and they also have some of their particles of the size approximately. On the other hand, looking at K9, it has a different pH (4) and their particles are much larger (not even the same order of magnitude) than the particles of the other samples. The same happens with density, for example the sample of K9 is denser than that of K10. At first this could sound strange, since they have the same composition and they have been synthesized in the same way, but the reason of this change in density is the particle size. While K9 has Cu nanoparticles of 4,4 μm, K10 has the 50% of Cu nanoparticles of 14,33 nm. This difference could lead to the difference in density of both samples.

In addition, as a result of the huge size of Cu nanoparticles obtained in DLS studies and in order to ensure that this value was correct, we have used SEM-EDS results. From them, it has been made a calculation of the particle size, and this calculation has shown that indeed the large particle size obtained in DLS studies was correct and, therefore, that K9 sample has huge Copper nanoparticles.

Talking about antibacterial activity and relating it with time, concentration of nanoparticles and nanoparticle size; particle size of ZnO nanoparticles play important roles in the antibacterial activity. ZnO nanoparticles antibacterial activity is size dependent, however, this dependency is also influenced by concentration of NPs. Larger surface area and higher concentration are accountable for ZnO nanoparticles antibacterial activity. ZnO nanoparticles of smaller sizes can easily penetrate into bacterial membranes due to their large interfacial area, thus enhancing their antibacterial efficiency. Controlling ZnO nanoparticles size was crucial to achieve best bactericidal response, and ZnO nanoparticles with smaller size (higher specific surface areas) showed highest antibacterial activity. This is why K3 antibacterial activity is considerably low; looking at DLS studies, it is shown that the nanoparticle size is quite large, so that the particles are not able to penetrate easily into bacterial membranes and their antibacterial efficiency falls. Having analyzed the behavior of K3 sample in presence of microorganism and how its nanoparticles have acted to slow this growth, there are some conclusions we can draw out of here; the first one is probably the clearest

one and is that the best time to let the microorganism in contact with the sample is 30 minutes. This is the time in which it is observed the lowest growth of microorganism and therefore the highest rate of growth inhibition of the ZnO nanoparticles. The best concentration of ZnO varies depending on the time. Furthermore, analyzing the concentrations of ZnO, it is seen that for short periods of time (5, 15 minutes), the concentrations that show better antibacterial activity are the lowest ones (1,2 and 3%), while for long periods of time the concentrations that show highest antibacterial activity are the highest ones (4 and 5 %). This does not make much sense, since a large number of studies investigated showed than increasing the concentration of ZnO, increases the antibacterial activity. One reason to explain this behavior is the presence of organic matter in K3 sample, which could have acted as a source of carbon for the microorganisms, so it can be a feed for them. In this case the action of ZnO is not strong enough for overcome this feeding properties of the composition. For this reason, when there is not enough time for ZnO to overcome this feeding, the Petris with higher concentration of ZnO NPs appear with higher concentration of microorganisms. However, for long periods of time, ZnO is able to get over this feeding and inhibit the growth of the microorganism with zinc oxide nanoparticles. All this leads to a greater concentration of microorganism in Petris with more concentration of ZnO nanoparticles at short period of times and therefore, a low concentration of microorganisms in Petris with high concentration of ZnO nanoparticles at long periods of time.

Among the others metal nanoparticles, the recent interest in the Cu nanoparticles is propelled by the possibility of exploring them as antimicrobial agent. Even today, the exact mechanism of antimicrobial action of the Cu NPs remains unknown. The general view seems to be a combination of several factors: releasing Cu^{2+} ions, their penetration and disruption cell membrane and biochemical pathway by chelating cellular enzymes and DNA damage. Based on such studies, leading to the ability that NPs in the range of 1–10 nm showed greater interaction with bacteria, which depends on as much as free surface to liberate ions, it becomes desirable to synthesize small and bare NPs [14]. Having analyzed the behavior of K9 sample in presence of microorganism and how its Copper nanoparticles have acted to slow this growth, there are some conclusions we can draw out of here; the first one is probably the clearest one and is that K9 shows much better results for short times of contact (5 and 15 minutes) than for long time of contact (30, 60 minutes). Nevertheless, it has great results in every experiment, regardless the time of contact between microorganisms and the suspension. Moreover, talking about concentrations, in the case of K9 sample, it has exactly what theory says; for higher concentrations of Cu NPs, it presents lower concentration of microorganisms, and therefore a really high inhibition of growth rate. This may seem odd at first, because the particle size is extremely large; this could present a problem when it comes to penetrating the membranes of the microorganisms and hindering the antimicrobial activity. However, this is not the case and it has a very good efficiency in terms of antimicrobial activity, as well as high growth inhibition rate.

Analyzing now K10, the first conclusion we can draw, is that it happens the opposite than in K3; for short periods of time (5, 15 minutes), the concentrations that show better antibacterial activity are the highest ones (100 and 200 ppm), while for long periods of time the concentrations that show highest antibacterial activity are the lowest ones (12,5, 25 and 50 ppm). In fact, for short times, it presents very good results in terms of growth inhibition rate, as for high concentrations the inhibition of

microorganisms is practically complete. However, when the contact of microorganisms with the suspension overcome 15 minutes, the highest concentrations start to exhibit high concentration of microorganisms. Therefore, for long periods of time, as the concentration of Cu NPs increases, the efficiency of the antimicrobial activity decreases, which is exactly the opposite of what it is said on the theory. Once again, what happens is just the presence of organic matter in K10 sample, which could have acted as a source of carbon for the microorganisms, so it can be a feed for them. In this case the action of Cu is not strong enough for overcome this feeding properties of the composition. About the size of Copper nanoparticles in K10 and their effect on the antimicrobial activity, it is difficult to assess this relationship due to the different sizes of the particles of this sample. But in general terms, the particles have small-medium size, which lead to a better efficiency on antimicrobial activity, due to their facility to penetrate in the membranes of the microorganisms and debilitate them.

Comparing now the three samples between them, the main conclusion is that the best sample is K9, because of its high inhibition of growth rate and therefore the high efficiency at antibacterial activity. It is the only sample that, having organic matter has overcome the power of microorganisms to be fed by this matter. Besides, the colonies created in K9 are less dense than those created in K3 and in K10. In fact, K10 and K9 should have behaved in the same way in presence of microorganisms, as they have been synthesized in the same way, except for the conditions; K10 was synthesized at 60ºC during 10 minutes while K9 was synthesized at 80ºC during 30 minutes, that could be the reason of their difference in behavior, and therefore in their antimicrobial activity. In general terms we can conclude that, in this experiments, they have been more efficient Cu nanoparticles than ZnO nanoparticles. It is important to highlight that although for short periods of time, ZnO nanoparticles have not been able to overcome the feeding of organic matter by the microorganisms, they beat this feeding when they have been given a little more time. K10 directly has not been able to overcome this feeding in any way, thus obtaining unreliable and meaningless results. In this sense we can conclude that the worst sample is K10 and that is probably because of how its Cu NPs have been synthesized. Possibly with more time and temperature in the synthesis, it would have reached the same good results as K9.

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