BIOSYNTHESIS OF SILVER NANO PARTICLES BY LEAF EXTRACT OF EUCALIYPTUS CINEREA AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITY

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ABSTRACT

In this work, biosynthesized silver nano particles, AgNP, were obtained using aqueous leaf extract of the Eucalyptus cinerea (Ec). The optimal ratio for silver nano particle biosynthesis, was the combination of 10 mL of the extract with 20 mL of a 5 mM AgNO3 solution. An analysis by Fourier transform IR spectroscopy of the AgNP was carried out in order to examine the possible reducing groups of the silver ion. The size of AgNP was studied using dynamic light scattering spectroscopy. The data analysis by Dynamic Light Scattering, DLS, revealed that the particle size distribution of AgNp in solution were between 10 and 60 nm and its average size 14 nm. The AFM image revealed that AgNp were spherical covered by extract matrix and size average of 90nm. The biosynthesis of silver nano particle was monitored continuously by UV-VIS spectrophotometric analysis. In the visible spectrum of AgNP dissolution,was observe the appearance of absorption peak at ~450nm, as a result of its surface Plasmon resonance. The antimicrobial activity of AgNP was evaluated against Bacillus cereus and Salmonella thiphymurium by the agar diffusion method. Antibacterial activity was obtained against B. cereus and S. thiphymurium; the activity varied according to the concentration, showing the appearance of inhibition halos at 100% concentration of AgNP in both microorganisms.

KEYWORDS

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1. Silver Nanoparticles, 2. Biosynthesis, 3. Antimicrobial Activity, 4. Eucalyptus Cinerea leaf extract, 5. Pathogenic Microorganisms

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INTRODUCTIÓN

The production of silver nano particles (Ag NP) from plant extracts has been a subject of great interest and study in recent years(Sidiq, 2018, Rayoriya, 2017, Ahmed, 2016; Reddy, 2015, Schrofel, 2014). This due to the multiple applications of this type of nano particles as anti-microbial, anti-viral y anti-larvicidalagent (Escárcega-González,2018; Erjaee, 2017 ; Stoyanova, 2016; Benakashani F, 2016; Banala, 2015; Ali, 2015; Arokiyaraj S., 2014; Mohamed , 2014; Shirmohammadi , 2014; Ghahfarokhi, 2014; Sila, 2014; Rajawat, 2012;González, 2017; Mohanta, 2017; Ramanibai , 2016; Velayutham, 2013; Marambio, 2010).

The biosynthesis of nano-particles is based on the use of biological entities such as micro-organisms, plant extracts and biomass of plants, which possess antioxidant and / or metal-reducing capacities (Ahmed, 2016).Its value is centered on the power to generate nanoparticles with additional properties conferred by secondary metabolites generated in plants.
The exact mechanisms of antimicrobial activities of silver nanoparticles are still under investigation and is a well-debated topic. Its antibacterial activity starts byAg^{+} ions releasing into the aqueous solution from AgNP partial oxidation. These ions and small nanoparticles interact with the plasma membrane, disrupting cellular functions, including permeability and respiration, and ultimately leading to lysis(Kotakadi 2015; Schröfel 2014; Sila 2014; Agnihotri 2014; Das 2014; Marambio 2010; Lok 2006; Sondi 2004; Dibrov 2002) .Also by interaction with DNA, enzymes and cytoplasmic proteins(Le 2010; Morones2005¸Hengge-Aronis,1996)and by the release of free radicals from silver nanoparticles(Kim2007).Free radicals can attack membrane lipids followed by their dissociation, damage and inhibition of the growth of microbes(Danilczuk 2006; Amro , 2000). In any of the mechanisms proposed by the different authors, AgNp cause cell lysis and subsequent death, ensuring the presence of a bactericidal effect(Salahuddin ,2018; Flores, 2014).

On the other hand, many of the plants that are used for the biosynthesis of NP Ag also have antimicrobial activity(Ahmed 2016; Ayepola 2008). Hence, there is a synergy between the properties of the extract of the plant and the nanoparticles obtained. In particular different species of Eucalyptus have been used in the production of AgNP (Sila 2014; Abd El-Rahman 2013), thisplant is native to Australia, belongs to the family Mirtácea and is known for its medicinal and therapeutic properties.In this work, the species of Eucalyptus

cinerea (Ec) cultivated in Venezuela was used. This species constitutes one of the 900 Eucalyptus species and sub-species. It has been reported that the main constituents of Ec's essential oils are 1,8-cineol (79.18%) and α-pinene (4.08%), showing a high antimicrobial activity against *Listeria ivanovii* and *Bacillus cereus* and in lesser amounts against *Escherichia coli, Staphylococcus aureus and Enterococcus faecalis*(Sebei 2015).

The World Health Organization reports that nearly 50,000 people die every day from diseases caused by bacterial pathogens, being the main cause of premature death, causing 33% of total annual deaths. (Disease,2004). Silver nanoparticles can be used as effective growth inhibitors in several microorganisms, making them applicable to various medical devices and antimicrobial control systems and considering low demand of antimicrobial agents to deal with infectious diseases caused by bacteria who happen to be resistant to antibiotics, in this study the AgNP biosynthesis was made from the extracts of leaves of*Eucalyptus cinerea*.

2. MATERIALS AND METHODS

2.1 Materials

Silver Nitrate ($AgNO_3$) was supplied by Sigma Aldrich. 18 MΩ deionized water was used in the performance of all the experiments. The *Eucalyptus cinerea* was collected from the Galipan population located on the northern slope of Cerro el Ávila, in Vargas state, Venezuela.

2.2 Preparation of Aqueous Eucalyptus Cinerea Leaf Extract

The collected leaves were washed with abundant tap water in order to remove the impurities. Afterwards they were left to air dry for a week and once dry, they were cut into small pieces and treated by grinding by a ball mill until a fine powder was obtained. 1 gram of leaf powder was transferred into the200 mL beaker containing100 mL distilled water. The solution is placed in ultrasound for 30 minutes maintaining a temperature of 60 ° C. Allow to cool and filter using a cotton ball. The extract is stored in a refrigerator at 4ºC for later use.

2.3 Biosynthesis of silver nanoparticles

For the production of the silver nanoparticles, an optimization of aqueous extract volume was made,initially extract different volumes, 5 to 40 ml were added to the 40 ml aqueous solution of 2 mM $AgNO_3$. These mixtures were made in a 250 mL vial, which is placed in a water bath under ultrasound for 30 minutes and at 40ºC, then incubated in a sand bath at 50ºC for 30 minutes more. The formation of the silver nanoparticles occurs when the color change of the amber yellow solution is observed to a permanent dark brown color. After observing this color change, the solution cools, is left to rest and proceeds to the subsequent characterization.Then the effect of precursor salt solution on extract was evaluated by varying $AgNO_3$ concentrations (1 and 5 mM).

2.4 Ag NPs Characterization

The particle size distribution was measured with a home device designed in laboratory, based on the technique of dynamic scattering of light. The equipment consists of a He-Ne laser of wavelength: 635 nm, two polarizers to attenuate the laser beam, a lens to focus the beam on the sample and a photomultiplier placed at a variable angle with respect to the incident light that converts it into electric current. The working conditions were: laser wavelength 630 nm, angle 90 °, temperature 298 K, refractive index 1.330 and viscosity 1.0020 cp, these last two values are characteristics of the solvent, in this case water .Atomic Force Microscopy (Dimensions Edge, Bruker) was used to determine the size and morphology ofAgNPs.The optical properties of surface Plasmon resonance, SPR, of the silver nanoparticles were monitored by UV-Visible absorption, using a UV-Visible Ocean Optics spectrophotometer, model. LS-I and data acquisition was obtained with the OOI Base32 program, version 2.0.1. Infrared spectra were acquired in a Nicolet instrument model Magna 750 Series II, operated at Fourier transformed mode. A spectral interval of 4000-400 cm^{-1} was used with a resolution of 4 cm^{-1} and a scan average of 64 scans.

2.5 Antibacterial Activity

The anti bacterial activity of AgNps obtained from the leaf extract of *E. cinerea*, was determined against *Escherichia coli, Bacillus cereus* and *Salmonella thiphymurium*, using the agar diffusion method. The trial consisted in the use of a soy nutritive broth (ATS) to cultivate the bacterial strains, after verifying the adequate growth of the microorganisms under study, plaques were inoculated in duplicate for each microorganism, planting 100 µL of the microorganism grown in nutritive broth on the surface of a plate with ATS.Subsequently sterile 5 mm diameter Whatman No. 1 paper discs were embedded in 10 µL of *E. cinerea* leaf extract, 10 µL of Ag NPs and 10 µL of Gentamicin (25 µg / mL) respectively, placed in a serial order within each plate and taken to incubation for 24 hours at 35 ± 2 ºC. Finally, the zone of inhibition was measured in millimeters through the use of a vernier (Biosynthesis of AgNPs using Carica Papaya peel extract and evaluation of its antioxidant and antimicrobial activities, 2016). The assay was performed in triplicate.

3. Results

Figure 1 shows the UV-Visible spectrum of the series of solutions of NP Ag-Ec, prepared using different volumes of the aqueous extract of eucalyptus and a fixed volume of 40 mL of a solution of 2 mM AgNO3, characteristic surface Plasmon absorption band was observed at 350 -500 nm, with a maximum absorption around 440 nm. An increase in the absorbance of the solutions of the nanoparticles was observed as the volume of extract increased; this is due to the fact that there are more reducing groups that interact with the Ag + ions and therefore a greater amount of metallic silver nanoparticles are formed (Kokila et al, 2016).

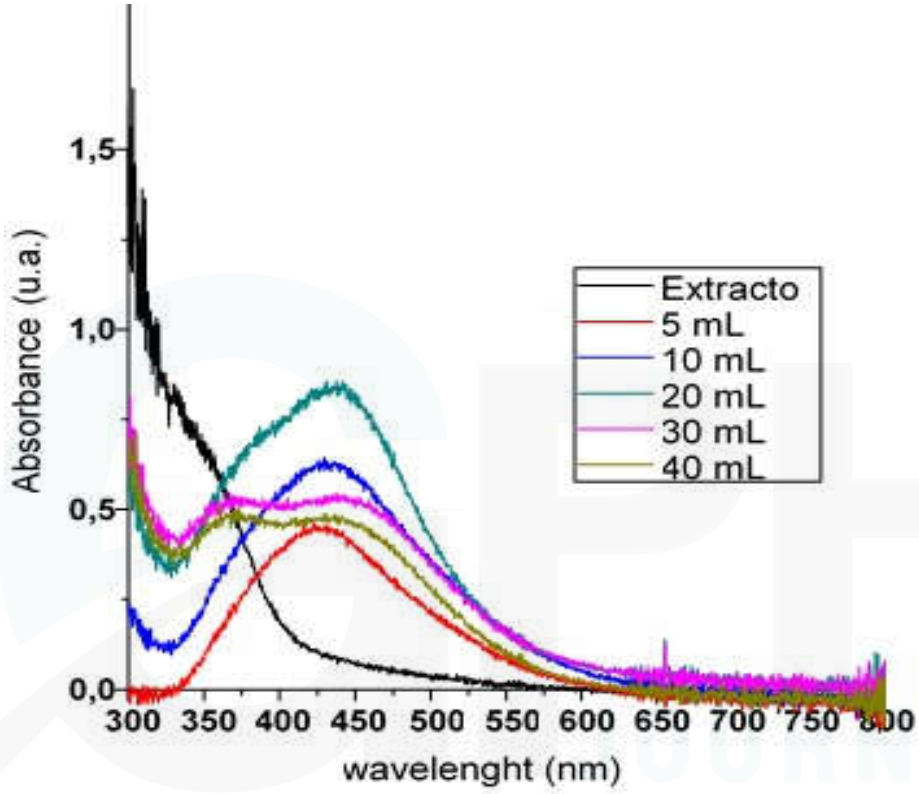

Figure 1. UV-Vis Spectra of AgNPs, using different extract volumes of E. cinerea at range betwen 5 y 40 ml.

However, at a volume of 30 mL of extract a broadening in the band is observed and the absorbance intensity decreases; which suggests that the nanoparticles are added and thus increases in size. The model of the surface plasmonic resonance in spherical nano-particles, according to the quasi-static theory (Kerker, 1982, Mie, 1908), establishes that the cross section of absorption is directly proportional to the volume of the particle while the dispersion depends on the volume square (Huffman, 2007). Therefore, the smaller the particle size, the absorption predominates over the superficial plasmon; while for large particles dispersion predominates and widening of the band occurs (Brongersma, 2007). Based on these results, the optimal extract volume for the synthesis is 10 mL.

Figure 2 shows the UV-Vis spectrum of the series of solutions of AgNPs obtained, when we have varied the $AgNO_3$concentration from 1mM to 5 mM and react whit afixed extract amount of 10 mL. The SPR band is observed between 400 and 600nm, with a characteristic maximum around 450nm. The increase in the concentration of the precursor salt produces an increase in the absorbance of the solutions of the nanoparticles, this due to the greater amount of Ag^+ ions available for its reduction to metallic silver.

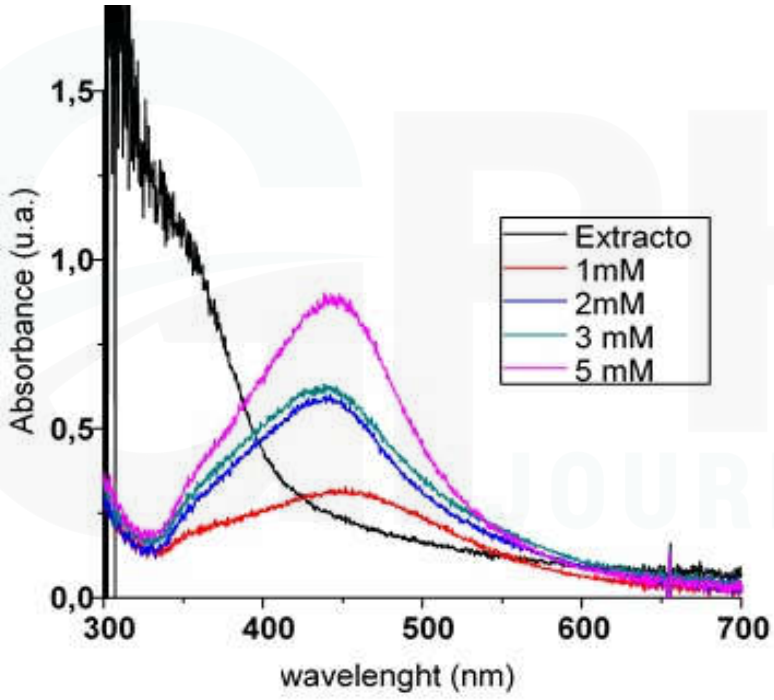

Figure 2. UV-Vis Spectra of AgNPs, varying $AgNO_3$ concentrations (1 to 5) mM whit an extract volume of 10 mL.

nanoparticles does not differ greatly as the concentration of $AgN0_3$ changes. The solutions of the nanoparticles presented a narrow size distribution between 10 and 60 nm, choosing as AgNO3 optimal concentration 5 mM, due to this concentration the highest absorbance was obtained and the average size was 14 nm. Similar results have been previously reported, reiterating that the extract content and the concentration of the precursor salt exerts an important effect in the size control of the nanoparticles (Kokila, 2016).

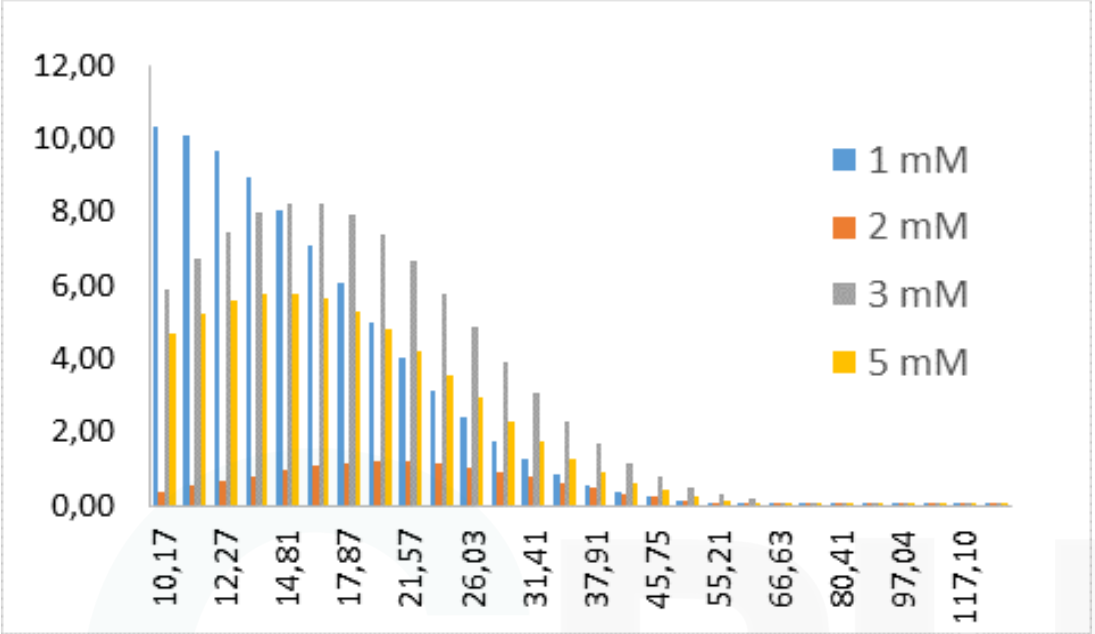

Figure3 Effect on Size Distribution of AgNPs synthetized by E. Cinerea using $AgNO_3$concentration at range 1 to 5 mM.

The AFM image of AgNps is shown inFig. 4. Direct observation of the image revealed that the size of the AgNPs was in the order of 90 nm. The particles appeared to be spherical in shapeand they are covered byleaf extract matrix. The size distribution of the AgNPs was shownin the figure, which clearly indicates that the majority of the AgNPs fell in the average range of 70–100 nm.

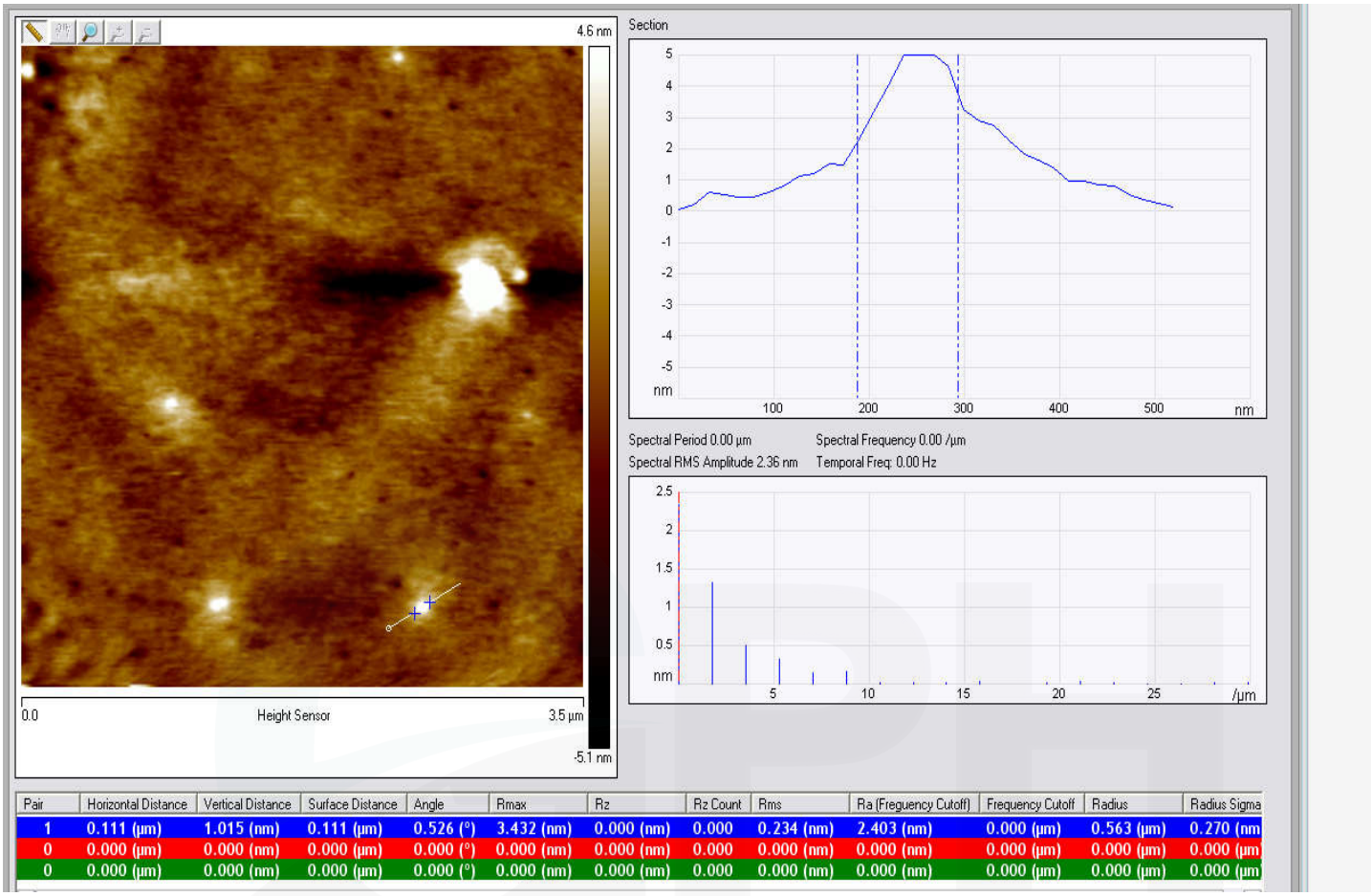

Figure4Image AFM of synthetized AgNPs by E. Cinerea using $AgNO_3$ concentration at 5 mM and 10 ml extract volume.

The FTIR spectra of the AgNPs is show in figure 5. The peaks close to 3000 cm-1 correspond to O-H stretchingcorresponding to alcohols and / or phenols that suggests the presence of polyphenols in the Ag NPs. The band that appears at 1624 cm-1 can be of an amide bond coming from the carbonyl groups C = O of a protein (Banala, 2015) and the peak at 1527 cm-1 is characteristic of the C = C bond corresponding to groups alquenos, the band at 1065 cm-1 corresponds to the C-O bond of phenolic acid and CN vibrations from amines. The phytochemical compounds with the functional groups C = O and C = C must participate in the reduction of the silver ion to metallic silver. These results suggest the role of the phytochemicals present in the aqueous extract of *Eucalyptus cinerea* as reducing agents of the silver ion and as stabilizers of the Ag nanoparticles (Banala, 2015, Velayutham, 2013, Abd El-Rahman, 2013).

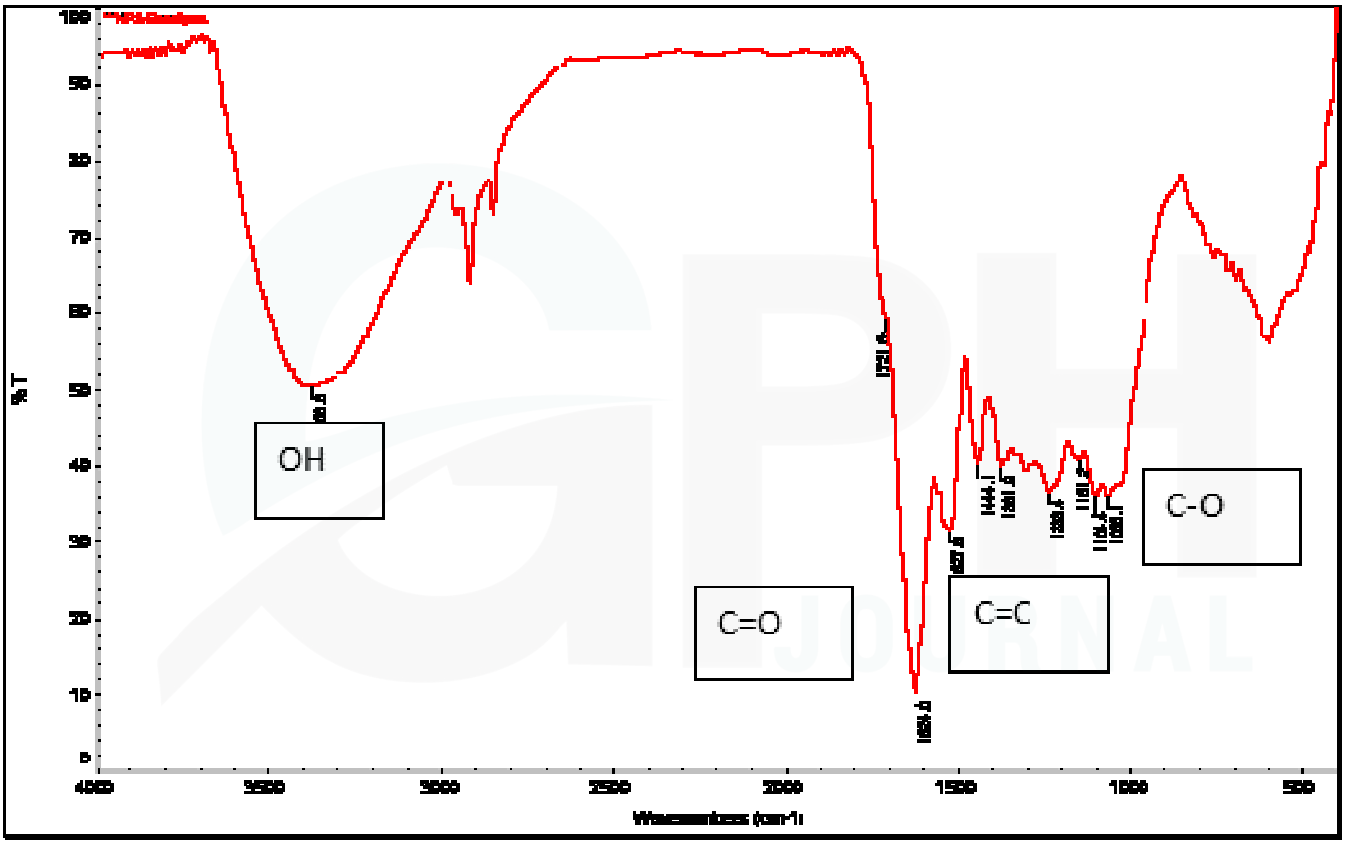

Figure5 FTIR Spectrum of AgNPs synthetized by E. Cinerea.

The nanoparticles AgNPs, showed antimicrobial activity against *B. cereus* and *S. Thiphymurium* (Table 1), this behavior can be attributed to the binding of AgNPs, to the surface of the cell membrane of the microorganisms, canceling out the permeation and the respiration of the cells (Siddiq, 2018). However, the activity varied according to the concentration, finding statistically significant differences ($p < 0.05$) regarding the inhibitory activity, when comparing the different concentrations evaluated of the nanoparticles.Table 1 shows the appearance of inhibition halos at a concentration of 100% NP Ag-Ec in both microorganisms, while the concentrations of 6.90 and 13.80% did not show antimicrobial activity, there being no statistically significant differences ($p \geq 0.05$) between both concentrations. For its part, the extract only showed no antimicrobial activity and gentamicin sulfate showed the highest measurements of haloes, used as controls, showing both statistically significant differences ($p \geq 0.05$) with respect to NP Ag-Ec.

In this study, the antimicrobial activity is due only to the Ag-Ec NPs and their concentration, where the toxicity of the silver nanoparticles against the microorganisms, is confirmed by the variation of the size of the inhibition halo, with which the constituents of the extract only intervene in the obtaining of the metallic nanoparticles. It was observed that the higher the concentration of nanoparticles, the higher the activity, this trend has been previously reported in the literature (Seong 2017, Ali 2015, Banala 2015, Ghahfarokhi1 2014, Shirmohammadi 2014, Sila, 2014). Sondi attributes this tendency to the fact that silver nanoparticles form "pores" or "holes" in the cell wall, accumulate and penetrate the bacterial cells causing their death (Sondi, 2014).

Table 1 Antimicrobial activity of AgNPs and *Eucalyptus cinérea* extract againts *Bacillus cereus*,*Salmonella Thiphymurium.*

Especie	Concentration (%)	Halo de inhibición (mm)	
		B. cereus	*S.Thiphymurium*
Extracto	2,00	0,00±0,00aA	0,00±0,00aA
Ag NPs	6,90	0,00±0,00aA	0,00±0,00aA
	13,80	0,00±0,00aA	0,00±0,00aA
	100	1,00±0,07bB	1,10±0,14bB
Sulfato de gentamicina	0,0024	1,50±0,07cC	2,75±0,35cC

The values represent the average of two determinations ± the standard deviation. The extract alone and gentamicin sulfate were used as controls. Different lowercase letters in the same column represent statistically significant differences ($p < 0.05$) for the inhibitory activity with respect to the concentrations for the same microorganism. Different capital letters in the same column represent statistically significant differences ($p < 0.05$) regarding the inhibitory activity for a given microorganism between nanoparticles.

Although the mechanisms of antimicrobial activity of Ag Nps may be ambiguous and not well defined, there is experimental evidence that the main interaction between Ag Nps and bacteria is by electrostatic attraction between the (positive) nanoparticles and the cells of the bacteria (negative) (Siddiq 2018; Stoyanova 2016) (Siddiq, (2018)), (Stoyanova D., 2016). As a greater quantity of Ag Nps and depending on their size, they can adhere more easily to the cell wall and even penetrate it, increasing in this way the inhibition in the growth of bacteria. However, the specific response of each bacterium depends on its metabolic characteristics (Pazos-Ortiz, 2017, Banala 2015, Berton 2014). Other factors such as the shape and coating of the particle also influence the mode of action of the silver nanoparticles on each microorganism (Xiu 2012, Hajipour 2012, Laban 2010, Kawata 2009, Fabrega 2009).

4. CONCLUSION

In this study, the green synthesis of AgNPs was carried out, using the aqueous extract of the leaves of the Eucalyptus cinerea plant. The nanoparticles exhibited an average size of 14 nm, showing a dependence in the control of particle size with the amount of extract and the concentration of the precursor salt. Infrared analysis by Fourier Transforms confirms the reduction of silver ions by several functional groups. UV-Visible spectroscopy confirms obtaining AgNPs. The nanoparticles AgNPs showed antimicrobial activity against B. cereus and S. Thiphymurium, the activity varied according to the concentration, showing the appearance of halos of inhibition at a concentration of 100% of NP Ag-Ec in both microorganisms.

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