SÍNTESE DE NANOPARTICULAS DE PRATA FUNCIONALIZADAS COM EXTRATO AQUEO (*Burseragraveolens*) E AVALIAÇÃO ANTIMICROBIANA EM *Escherichia coli, Staphylococcus aureus E Klebsiellapneumoniae*

SYNTHESIS OF SILVER NANOPARTICLES FUNCTIONALIZED WITH AQUEOUS EXTRACT (*Burseragraveolens*) AND ANTIMICROBIAL EVALUATION IN *Escherichia coli, Staphylococcus aureus AND Klebsiellapneumoniae*

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Received 05 December 2017; received in revised form 16 December 2017; accepted 20 December 2017

RESUMO

Atualmente, a resistência aos antibióticos tornou-se um problema para o tratamento de doenças infecciosas. Neste estudo, as nanopartículas de prata de 16 ± 2 nm foram sintetizadas utilizando o extrato aquoso de Palo santo (*Bursera graveolens*), uma planta conhecida por sua ação biocida, para determinar seu efeito antimicrobiano sobre *Escherichia coli* e *Staphylococcus aureus*. Diferentes concentrações de nanopartículas (100 until1000 ppm) foram avaliadas nos tempos de contato de 1, 2, 5 e 10 minutos para 1500 e 15000 UFC / mL. Além disso, para determinar a eficácia, foram realizados testes de difusão de disco utilizando várias concentrações de nanopartículas (500-1000 ppm) em *S. aureus* e *K. pneumoniae*. De acordo com os resultados do efeito antimicrobiano para *E. coli,* a a eficácia dasnanopartículas pode ser estabelecida em todos os tempos de contato para 1500 CFU de 800 ppm e para 15000 CFU de 400 ppm. Com *S. aureus*, a inibição total foi mostrada a partir de 800 ppm em 1500 CFU a 5 minutos de contato e entre 500-1000 ppm com 15000 CFU no minuto um. Os testes de *S. aureus* e *K.pneumoniae* mostraram sensibilidade com nanopartículas. A média de susceptibilidade para *S. aureus* apresentou menor inibição entre 500-600 ppm e a mais alta em 700-800 ppm. A susceptibilidade média para *K.pneumoniae* é mais constante em termos de inibição a 600-1000 ppm. Este estudo demonstrou o efeito antimicrobiano das nanopartículas de prata sintetizadas nas condições especificadas.

Palavras-chave: *nanopartículas de prata, palo santo, Burseragraveolens, E. coli, S. Aureus, K. pneumoniae*

ABSTRACT

Currently, resistance to antibiotics has become a problem for the treatment of infectious diseases. In this study, silver nanoparticles were synthesized using the aqueous extract of Palo santo (*Burseragraveolens*), for its biocide action, in order to determine its antimicrobial effect on *Escherichia coli* and *Staphylococcus*

DOI: 10.52571/PTQ.v15.n29.2018.282_Periodico29_pgs_282_291.pdf

aureus. Different concentrations of nanoparticles (100 until1000 ppm) were evaluated at contact times of 1, 2, 5 and 10 minutes for 1500 and 15000 CFU/mL. Additionally, to determine effectiveness, disc diffusion tests using various concentrations of nanoparticles (500-1000 ppm) were performed on *S.aureus and K. pneumoniae.* According to the results of antimicrobial effect for *E.coli*, the effectiveness of nanoparticles can be established at all contact times for 1500 CFU from 800 ppm and for 15000 CFU from 400 ppm. With *S.aureus*, total inhibition was showed from 800 ppm in 1500 CFU at 5 minutes of contact and between 500-1000 ppm with 15000 CFU at minute one. S. aureus and *K.pneumoniae* tests showed sensitivity with nanoparticles. The average of susceptibility for *S.aureus* had the lowest inhibition between 500-600 ppm and the highest at 700-800 ppm. The average susceptibility for *K. pneumoniae* is more constant in terms of inhibition at 600-1000 ppm. This study demonstrated the antimicrobial effect of silver nanoparticles synthesized under the specified conditions.

Keywords*: Silvernanoparticles, palosanto, Burseragraveolens, E. coli, S. Aureus, K. pneumoniae*

INTRODUCTION

The native *Bursera* (in *Burseraceae*) comprises 40 species distributed in tropical forests, with some species in xeric shrublands, diversifying along the pacific slope, Central America, north-western South America and mainly below 1700 m elevation in the south-western USA. Indeed, a few endemic species occur on the Galapagos and Revillagigedo archipelagos, whereas several endemic species are distributed extensively in Bahamas, Cuba, Jamaica, and Hispaniola.

The plant genus *Bursera* has been used as a model for biogeographical analyses because of its high species richness and large number of endemic species (ca. 100 species of trees and shrubs).

Their implications for its phylogenetic classification have been reviewed to hypothesize on the historical biogeography of tropical: Mexico and Galapagos (Espinosa, Llorente and Morrone, 2006).

In other cases, the resin samples from botanical species from Mexican *Bursera* were studied to determine their botanical origin related to triterpenic composition of archaeological Aztec objects (Weeks and Tye, 2009). Even its resin has been studied in less-know application to second generation ethanol in biorefinery. It is mostly-known for its essential oils and secondary metabolites used as perfumes and folk medicines.

Two species of Galapagos *Bursera* (also known as: *Palo Santo*, Spanish for *holy wood*) are generally recognized: the native *Burseragraveolens* (Bg) Triana & Planch (Kunth) and *Burseramalacophylla* B. L. Rob. The latter is categorized on the International Union for Conservation of Nature (IUCN) red list as vulnerable to extinction and consequence of its extremely restricted distribution. However, Bg is a recommended taxon as subspecies malacophylla (Weeks and Tye, 2009).

Bg species is a wild native tree and has been used as a remedy for gastric aches, liniment for rheumatism and sudorific (Morton, 1981) as well as for sweet and spicy ancestral odor and medicine (Ogata, 1989). The alcoholic extracts (e.g. rum liquor) in leaf and bark are applied locally for washing wounds and orally against asthma, diarrhea, calculi in the kidney (Bernal and Correa 1990). On the other hand it has a strong woody odor and has been used for incense by the indigenous people to prevent mosquito bites (Neiro, Oliveiro and Stashenko, 2010).

In the past year, Bg has aroused interest being that its aroma, and a broad range of applications including food additives, daily-used chemical products, tobacco additives, can be used as intermediates for the synthesis of dihydrogen mint and menthofuran and have held the attention of synthetic chemists (Wang *et al.,* 2017; Bilel et al., 2011).

Same phylogenetic reviews (Daly, Fine and Martínez, 2012) indicate *Bursera* is notorious for hybridizing and were colonized from Central America. Most species are restricted endemics which showed an apparent link between *Bursera* and *Commiphora*. Hence, it is possible that the active phytochemical was common. Some of its essential oils are rich in n-terpenes: mono (limonene derivatives) (Monzote *et al*., 2012), sesqui (juneols, eudesmane and germacrene derivatives) (Zviely and Boix, 2015, Yukawa *et*

al., 2004; Young *et al*., 2007) and tri (eoleanic and ursanic acid derivatives) (Robles *et al*., 2005); in oleoresins (elemicin derivatives], menthofuran) (Luján*et al*., 2012), lactone (Yukawa *et al*., 2006) amongothers. These active phytochemical allow for developing eco-friendly methods for the synthesis of biologically active silver nanoparticle by means of new-fangled mechanisms of reduction employing some plant extract indicated.

It is estimated that today about 320 tons/year of nanosilver are produced and used worldwide, even though it is a very minor component of overall silver volumes with ca. 20 Mtons production in 2016 (a 1% decline from the previous year) (Nowack, *et al.,* 2011). Silver nanoparticles (AgNPs), joined with Ti derivatives are the most biocompatible materials (Oshida, 2013). Conversely, other reviews showed that over the years, the AgNPs in water are toxic (Reidy *et al*., 20139), either because of its particle size, or the catalytic activity of the ions (eg. agglomeration/aggregation vs dissolution, superficial coating vs superficial charge, or internalization) (Larue *et al*., 2014). Despite the pros and cons, among the noble metal nanoparticles, they are present in many applications series due to simple and tunable synthesis routes and morphology.The noble nanoparticles are used as biosensors in forensic sciences (Marin *et al*., 2015)

The NPs green synthesis, eco-friendly speaking, includes chemical, physical and biological method leading no-toxic and non-impact environmental byproducts with non-expensive processes. Many approaches have been developed to increase demand for green nanotechnology: eg. Polysaccharide-stabilized (Morsi *et al*., 2017), phytoextract-precursors alkaloids (Gomathi *et al*., 2017), agricultural wastes (Ndikau *et al.,* 2017) among others.

Toxicity studies on pathogen opens a door for nanotechnology applications in medicine. Biological synthesis of metal NPs is a traditional method and the use of plant extracts has a new awareness for the control of disease, besides being safe and having no phytotoxic effects. The biologically synthesized silver nanoparticles using medicinal plants were found to be highly toxic against different pathogenic bacteria and fungi of selected species (Savithramma *et al*., 2011).

To the best of our knowledge, no articles were found evaluating the toxicity and synthesis of AgNPs green-synthesis using Bg phytochemical extracts. In this study, we report the synthesis of AgNPs by green synthesis process using Bg leaf aqueous extract as reducing agent. The final product characteristics were studied – optical, structural, surface morphology and elemental analysis. Along with these characteristics, the antibacterial ability against *E. coli*, *S. aureus* and *K. pneumoniae* was examined and reported. The Bg varieties used were the ones from the Galapagos Islands and those from the city of Manta which are the common specimens found within Ecuador.

MATERIALS AND METHODS

2.1. Preparation of Leaf Extract

The Bg leaves were randomly collected in the region of the Galapagos Islands (Santa Cruz) geographic coordinates: -0.686072, -90.363234 y -0.694677, -90.333429.

The Bg leafs were washed several times with distilled water to remove any impurity. They were then allowed to dry in an oven at 25 °C for 5 days. With the leaves completely dried they were crushed with mortar and pestle.The resulting product was collected in Falcon tubes which were stored at 4 °C.

The aqueous extractions were performed, taking 0.5 g of the frozen store precursor, mixing with 20 mL of dH_2O and constant stirring in Erlenmeyer flask at (60±1) °C for 1 h. The mixture was centrifuged at 2000 rpm (~2000 G) for 20 min. The supernatant liquid was filtered through Whatman 41 paper under vacuum. This aqueous fraction, used before for AgNPs synthesis, was stored at 4 °C.

2.2. Synthesis of Silver Nanoparticles

The method was optimized, by variation of phytoextract volumes, reaction (contact) times and temperatures, pH and precursor concentrations, analyzing the AgNPs stability formed. The optimal parameters were the usage of 20mL of $AgNO_3$ (1mM, Scharlau, analytical grade). It was added to 1mL of the aqueous extract of Bg, drop by drop. Adjusting the pH, 8 drops of NaOH Merck® (1%, v/v) were added with constant stirring for 60 min. at (60±1) °C. A naked-eye control to complete reduction

conversion from Ag^+ to Ag was the solution color change (from slightly yellow to dark brown).

2.2.1. Characterization of Synthesized Silver Nanoparticles

The AgNPs formation was verified from a pronounced UV-Vis peak around 400-450 nm (Ag plasmon resonance) being more pronounced for higher $AgNO_3$ concentrations, indicating that more nanoparticles density is generated. The *Agilent Technologies, Cary 60 UV-Visible spectrophotometer model* (resolution of 1nm, optical path length of 10mm within 300-800 nm range) was used. A 1:10 dilution with dH_2O was performed.

Additional FTIR-ATR experiments were under way to further characterize the phyto extracts (crushed and dried plant specimens), the AgNPs (standard alone) and its functionalization. The *Perkin Elmer spectrophotometer, Spectrum BX FTIR*, with ATR coupling (resolution of $4cm^{-1}$ at $4000\text{-}400cm^{-1}$ and 10 scans per sample with intervals of $2cm^{-1}$) was used. The vegetable extract and the nanoparticles obtained were observed, which were centrifuged and dried at 25 °C.

The STEM analysis of the colloidal suspension nanoparticles was performed in a FEG-SEM Tescan Mira 3 microscope using a voltage of 30 kV. TEM micrographs were obtained using a Philips CM-200 of 200 kV electron transmission microscope.

The SEM-EDX analysis was performed on a Phenom Pro X Scanning Electron Microscope equipped with EDX-detector operating at 10kV and Prosuite-EDS software.

2.3. Antibacterial Activity of Synthesized Silver Nanoparticles

The antibacterial activities of AgNps-Bg were carried out by modified method AOAC 960.09 with bacteria E. coli (ATCC 25922) and *S. aureus* (ATCC 25923) procured by Microbiologics®. This analysis was realized in the Laboratory of Microbiology, DISerLAB PUCE.

In this method, a sample of the AgNP-Bg is inoculated with a suspension of representative test microrganism obtained from, starting from the McFarland scale 0.5 (1.5 x 108 UFC / mL DO 0.08-0.10) until reaching the concentrations of 1500 and 15000 CFU / mL. In order to define optimal concentrations and contact times, the AgNPs-Bg sample was seeded in ten concentrations (100 until 1000ppm) with an inoculum of 1500 and 15000 CFU / mL at contact times of 1, 2, 5 and 10 minutes. Then 1mL of 10 concentrations of AgNPs-Bg (100 until1000 ppm) were poured with 1 ml suspension of test organism in test tubes from the previously seeded strain in BHI DIFCO®. This mixture was striated in the solid medium of TSA in four quadrants with 4 striae each on the surface of the agar. Each quadrant with a contact time of at 1, 2, 5 and 10 minutes and was finally incubated for 24 h at 35 to 37◦C. After incubation, the growth of the inoculated microorganisms in each quadrant varied. This experiment was repeated three times.

Additionally, to determine whether or not whole phyto extract were effective antimicrobial agents, disc diffusion tests using various concentrations (500-1000 ppm) were performed on Streptococcus sp. (CAMP and ATCC) and *K. pneumonia* (KPC and ATCC). Positive (precursor discs) and negative (phyto extract discs) control tests were also performed.Susceptibility patterns were determined by measuring the zone of inhibition (ZOI) of bacterial growth, in millimeters. This essay was realized at the Laboratory of Microbiology of UDLA.

RESULTS AND DISCUSSION

The formation of the AgNPs, the change of color of the solution from yellow to reddish-brown, was verified with the naked-eye when the pH medium becomes basic due to the reduction of precursor to silver colloidal nanoparticles. The characteristic UV-Vis band of Ag-SPR is between 400 and 450nm, so its spectrum was a very useful tool for its characterization. Its intensity was increased being more pronounced for higher concentrations or the precursor**,** indicating that more nanoparticles density was generated. Even more, Ag^+ were reduced and the AgNPS were increased, when the reaction time had progressed. This effect, due to the excitation of SPR, has been also reported in several studies carried out with plant extracts according to Ndikau *et al* (2017).

The AgNPs-Bg synthesis was optimized by modifying the parameters of aqueous extract volume, time, temperature, pH and the precursor

concentration.

Table **1** shows the summary of these parameters optimized together with UV-Vis spectroscopic result.

Table 1. *Optimal parameter (aqueous extract volume, contact time, temperature, pH and the precursor concentration) and their spectroscopic UV-Vis values*

	λ (nm)	Abs (au)	
V (mL) 1	406	1.220	
t (min) 60	405	1.229	
T (ºC)	60	404	1.246
pH	8	405	1.228
[P] (mM)	1	405	1.240

The UV-Visible spectra of Ag-SPR for these tests over five parameters can be seen in Figure **1** to **5**. Figure 5 indicates optimal parameters (Table 1)**.** In Figure 1 it can be observed that the optimum volume of aqueous vegetal extract, to form the AgNPs, with precursor concentration of 1mM concentration is only 1mL. When the phyto extract volume was increased, the intensity of the Ag-SPR decreased. As there are more reducing species in the reaction medium, which form more primary AgNPs, they favor aggregation, forming secondary AgNPs larger than expected. Another evidence of the aggregation was the presence of two isosbestic points (ca 370 and 410 nm) so the volume of the phyto extract was increased.

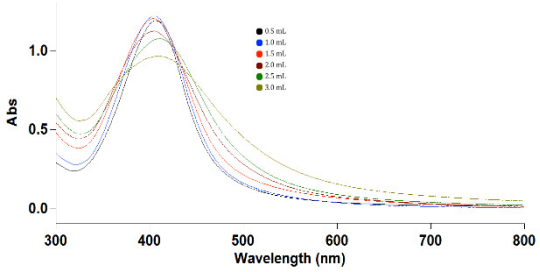

Figure 1. *Ag-SPR band with phytoextract volume variated (0.50-3.0 mL)*

As the reaction time and temperature was increased in Figure **2** and Figure **3**, the intensity of the Ag-SPR peak also increased, although the wavelength was almost invariant (405nm @ 60min and 60 °C). The clear presence of an isosbestic point with the variation of temperature, ca 450 nm, is indicative of either aggregation or some parasitic reaction that favors the formation of AgNPs. Another isosbestic point, burried in around 370 nm**,** would correlate with the one that appeared with the volume variation of the phyto extract.

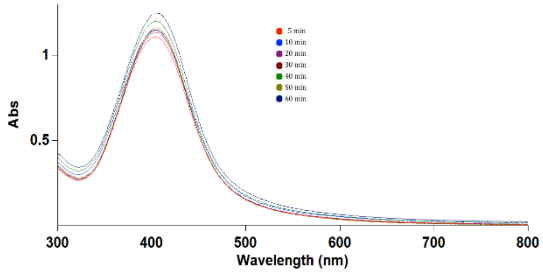

Figure 2. *Ag-SPR band with reaction time variated (5 and10-60 min)*

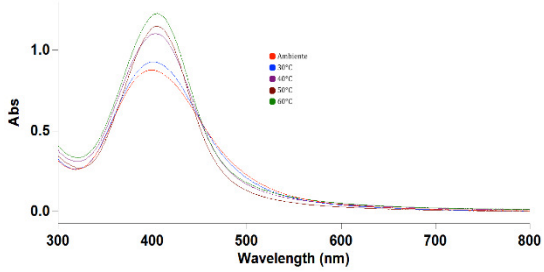

Figure 3. *Ag-SPR band with* temperature variated (21-60 °C)

The effect of pH on the formation of nanoparticles (Figure **4**) had a different tendency: at acid pH the reduction reaction is not effectively indicated as absorbance of residual Ag-SPR; situation contrary to basic pH, where the highest value appears at pH 8.

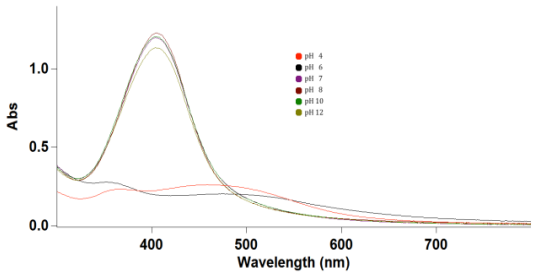

Figure 4. *Ag-SPR band with pH variated*

Finally, in relation to the concentration of the precursor reagent ($AgNO_3$) in Figure **5** the maximum absorption at 10 mM was observed, unlike lowest concentrations (0.1, 0.5 and 1.0 M),

where the lower absorbances indicated low concentration of AgNPs.

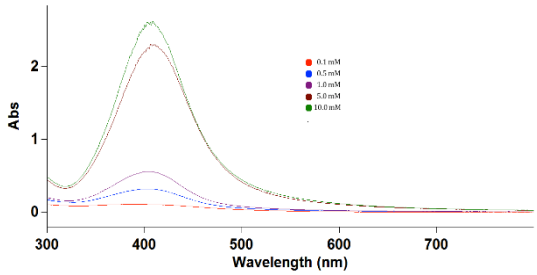

Figure 5. *Ag-SPR band with precursor concentration variated (0.1- 10 Mm)*

FTIR-ATR spectra showed an adequate organic covering of synthesized nanoparticles. In Figure **6** the precursor bands are showed. The widest one at 1269.5 cm^{-1} and followed by the other smaller band at 799.6 cm^{-1}, both of which could be attributed to the nitrate anion. They are somewhat shifted towards lower energies, as Coates (2000) established the group frequencies at 1380-1350 and 840-815 cm^{-1}; nevertheless, it is important to point out that both bands comply with the author's description in regard to broadness and intensity. The AgNPs and Bg bands (phyto extract) showed almost the same bands and no precursor, which indicated a successful coverage of AgNPs surface with active compounds. Considering previous reports from Yukawa *et al* (2004) and Schultz *et al* (2005), it is very likely that the phytoextract contains terpenoids and sesquiterpenoids (2359, 1600, 1030-1048 cm^{-1}) attributable to C=O, C=C and C-O-C, respectively. Peaks at 1602 and 3280 cm^{-1} may be indicative to the presence of –NHRR' amines, corresponding to the N-H stretching and bending, respectively. It is possible that these amines act as reducing agents onto phyto extract, successfully involved in AgNPs synthesis (Frattini *et al*, 2005).

The STEM and TEM micrographs of the AgNPs-Bg nanoparticles showed polydispersion (Figure **7**) which was indicative of the dual effect of the Bg extract as reducing and coating agent on the metallic surface. The average nanoparticle size was 15.92±2 nm.

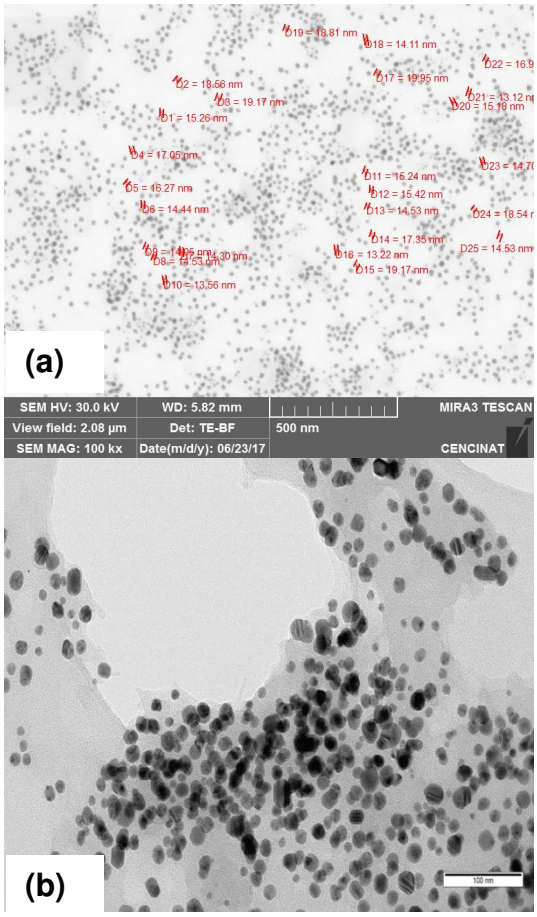

Figure 7. *Micrographs AgNPs-Bg 1M: STEM 100 kV (a) and TEM 200 kV (b)*

The SEM micrograph in Figure **8** shows the dispersion as bright points of AgNps-Bg powder and the EDX spectrum (its inset) shows the three characteristic peaks of the silver under the 4kV, the rest of the elements come from the other organic phyto extract.

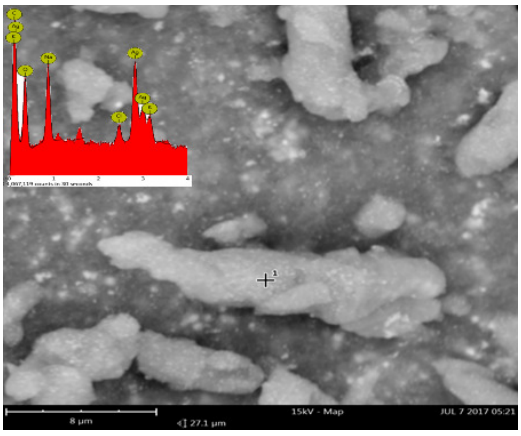

Figure 8. *Micrograph of AgNPs-Bg 1M by SEM 15 kV. Inset: SEM-EDX spectrum*

Figure **9** shows the results of each test. For the *S. aureus* and *K. pneumoniae* tested, all organisms showed sensitivity to the AgNPS-Bg (susceptibility pattern > 0.6 mm), and did not ZOI for the phyto extract paper disc (susceptibility pattern = 0.6 mm). If we consider that the antibacterial mechanism of action of the AgNPs-Bg is invariant in the two strains of each bacterium considered. It can be seen that the average for *S. aureus*, has two zones. The lowest inhibition 500-600 ppm and the highest 700-800 ppm. The average for *K. pneumoniae* is more constant in terms of inhibition at 600-700 ppm and 800-1000 ppm.

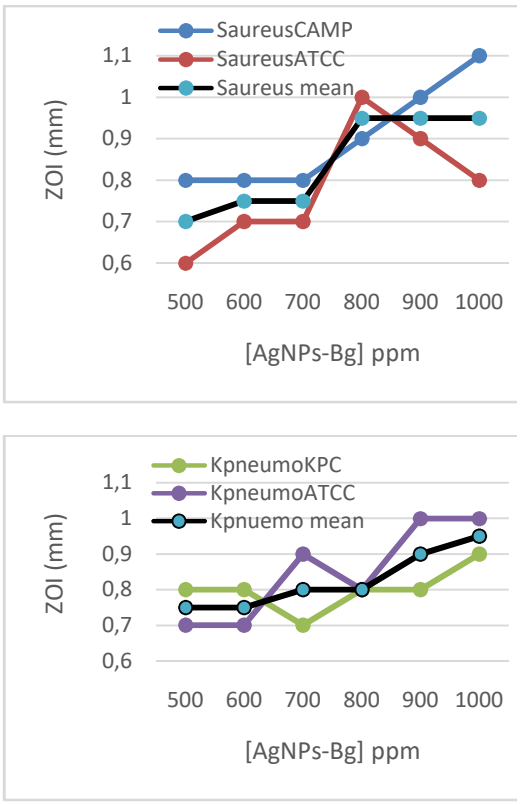

Figure 9. *Susceptibility pattern for S. aureus (a) and K. pnuemoniae (b) with AgNPs-Bg concentration variated*

From Table **2** at the end of this manuscript, values for *E. coli*, the effectiveness of the product faced; (the highest concentration value of AgNPs-Bg common) can be established at all contact times for 1500 CFU/mL to 800 ppm and for 15000 CFU/mL to 400 ppm. Conversely, the ineffectiveness of the product faced is 500 and 200 ppm for 1500 CFU / mL and 300 ppm for 15000 CFU/mL. On the other hand, some outliers can be found that should be attributed to the experimental limitation.

Finally, Table **3** for *S. aureus*, the effectiveness of the product faced can be established clearly at all contact times to 500 ppm for 15000 UFC/mL. This value corresponds with the zones of lowest and highest inhibition commented in susceptibility pattern (Figure 9a). The ineffectiveness of the product faced for 1500 UFC presents several outliers that make for all contact times non homogeneous. If contact times of 1, 2 min are considered standalone, a local effectiveness at 500 ppm and 600 ppm can be established which corresponds with the zone of lowest inhibition (ZOI>0.6 mm). For the 5, 10 min this local effectiveness is 800 ppm related to zone of highest inhibition (ZOI >0.9 mm).

The limitation of the study may be due to the lack of tests at lower concentrations than those used with the microorganisms evaluated, as well as more variation with the contact times tested. The samples of the product were manipulated and maintained until the test in optimal conditions covered by standardized protocols.

CONCLUSIONS

The AgNPs-Bg synthesis was optimized with aqueous phytoextract volume, reaction time, temperature and pH, and the precursor concentration parameters-variated. The obtained conditions can be seen in Table 1.

The characterization of AgNPs-Bg showed an adequate organic covering with the phyto extract selected (Bg), close optimal polydispersion (<size >=16±2 nm) and effective antimicrobial activity, seen of FTIR-ATR, electron microscopy and agar dilution well diffusion method respectively.

FTIR-ATR spectra comparison of precursor, phytoextract and AgNPs-Bg betrays the presence of bands with band of nitrate anion, sesquiterpenoids and secondary amines in accordance with other authors.

STEM and TEM µgraphs show said polydispersion, and SEM µgraphs and SEM-EDX spectrum reaffirm that morphology.

The effectiveness (1500 and 15000 UFC/mL) for *E. coli* were determined at around 800 ppm and 400 ppm. For *S. aureus* at 500 ppm and 500-800 ppm with each concentration

respectively. These limitations are directly related to the concentrations tested, future studies must be carried out varying the concentrations with lower microbial populations.

ACKNOWLEDGMENTS

This study was funded by Pontifical Catholic University of Ecuador with the Project N13438 (2016-2017). A special thanks to Dra. Svetlana Ivanova from the University of Seville for the TEM analysis, Dr. Alexis Debut from CENCINAT-ESPE for the STEM analysis and MSc. Carlos Bastidas from UDLA for sensitivity analyses with *S. aureus* and *K. pneumoniae.*

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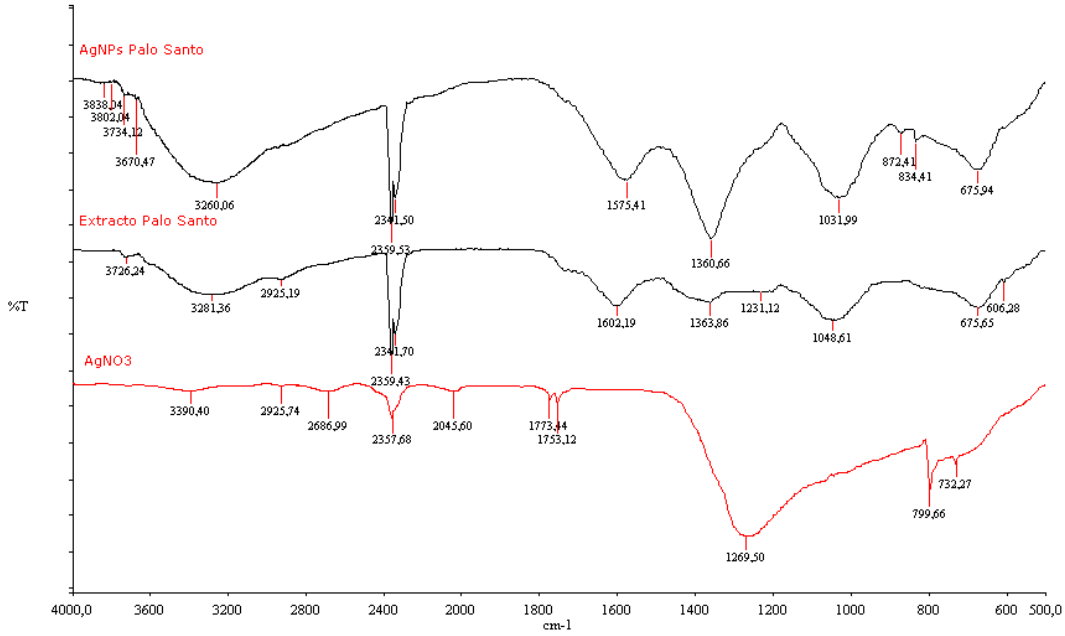

Figure 6. *FTIR-ATR spectra comparison of precursor, phytoextract and AgNPs-Bg (from bottom to top).*

Table 1. *Interpretation of antibacterial susceptibility test (G/I) for E. coli with the phytoextract (Bg), precursor concentration (P) and AgNPs-Bg at 100-1000 ppm(+100).*

E. coli				
1500 UFC/mL				
	1 min	**2 min**	**5 min**	**10 min**
G	Bg 100-300 500-600	Bg 100-300 500	Bg, P 100-200 500	Bg, P 100-200 500-700
I	P 400 700-1000	P 400 600-1000	 300-400 600-1000	 300-400 800-1000
15000 UFC/mL				
	1 min	**2 min**	**5 min**	**10 min**
G	Bg 100-300	Bg, P 100-300	Bg, P 100-200	Bg, P 100-300
I	P 400-1000	400-1000	300-1000	400-1000

Bg: phytoextract, P: [precursor]=1000 ppm, G: growth, I: inhibition.

Table 2. *Interpretation of antibacterial susceptibility test (G/I) for S. aureus with the phytoextract (Bg), precursor concentration (P) and AgNPs-Bg at 100-1000 ppm(+100).*

S. aureus

1500 UFC/mL				
	1 min	**2 min**	**5 min**	**10 min**
G	Bg 100-400 600 800	Bg,P 100-500 800-1000	Bg, P 100-300 500 700	Bg, P 100-300 500
I	P 500 700 900-1000	600-700	400 600 800-1000	400 600-1000
15000 UFC/mL				
	1 min	**2 min**	**5 min**	**10 min**
G	Bg 100-400	Bg,P 100-300	Bg, P 100-300	Bg, P 100-200
I	P 500-1000	400-1000	400-1000	300-1000

Bg: phytoextract, P: [precursor]=1000 ppm, G: growth, I: inhibition.