

Green synthesis: characterization and biological activity of silver nanoparticles using aqueous extracts of plants from the *Arecaceae* family

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ABSTRACT. This study proposes the preparation, characterization, and evaluation of the antimicrobial activity of silver nanoparticles (AgNPs). AgNPs were synthesized from the leaf extracts of plants from the *Arecaceae* family, which are abundant in the Amazon region. AgNPs were characterized using UV/Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), dynamic light scattering (DLS), and their minimum inhibitory concentrations (MIC) against the bacteria *Escherichia coli* and *Staphylococcus aureus*. AgNPs presented maximum absorbance between 420 and 430 nm, the mean diameter obtained by DLS ranged from 130.43 to 352.93 nm and the polydispersity index (PdI) ranged from 0.523 to 0.689. The surface charge measured by the Zeta potential was negative and ranged from -17.2 to -26.97 mV. FTIR analysis suggests that the phenolic compounds and/or proteins in the chemical composition of the plants studied may have been responsible for the reduction of Ag⁺ ions and stabilization of AgNPs. The morphology of AgNPs observed was largely spherical and presented some agglomerates. Transmission electron microscopy analyses showed polydispersed AgNPs without the formation of large agglomerates. The synthesized AgNPs presented homogeneity and rapid bioreduction. The concentration of AgNPs required to eliminate microorganisms by up to 90% was lower for Gram-negative bacteria (2.75 µg mL⁻¹) than for Gram-positive bacteria (21.75 µg mL⁻¹). In addition, AgNPs synthesized from plant species that are native to the Amazon proved to be promising, since they showed excellent antimicrobial activity against microorganisms of clinical interest.

Keywords: scanning electron microscopy; dynamic light scattering; antimicrobial activity; transmission electron microscopy.

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Introduction

Nanotechnology is a science that deals with the creation, manipulation, and applications of nanometer-scale materials (10⁻⁹ m) (Korbekandi & Iravani, 2012; Ghaedi, Yousefinejad, Safarpour, Khafri, & Purkait, 2015). Nanobiotechnology is an area that connects nanotechnology and biotechnology, and whose purpose is to improve nanostructures in biotechnological processes in order to transform systems and products (Akhtar, Panwar, & Yun, 2013). The great interest in "nano" materials is due to their physicochemical properties, which differ from materials at larger scales, and thus make it possible to use them in new applications (Ahmed, Ahmad, Swami, & Ikram, 2016).

Several methods are available for synthesizing metallic nanoparticles (NPs), and among the most reported is chemical reduction via reducing agents in order to stabilize the nanoparticles, however, many of these reducing agents are toxic and/or have high costs. In addition, this process requires the use of specific equipment and has a high consumption of energy (Cauerhff & Castro, 2013; Iravani, 2011). Biological organisms, such as plants, can be exploited as a substitute for chemical reagents that, besides being expensive, are potentially harmful to the environment.

The enormous biodiversity of plants is a factor that makes them attractive for the synthesis of AgNPs. Normally, the synthesis of silver nanoparticles (AgNPs) occurs through the bioreduction of silver by the components of the vegetal extract and characterized by the change in coloration of the solution. The phytochemical constituents, among them phenolic compounds, are recognized as potential stabilizers of

metallic nano particles (NPs) because each plant has a unique chemical composition (Aromal & Philip, 2012; Kulkarni & Muddapur, 2014; Haider & Kang, 2015).

The *Arecaceae* family includes several species that are employed in a number of applications, such as for lumber, as a food source, and in landscaping (Zambrana et al., 2007). In addition, this family presents a considerable level of phenolic substances (antioxidants) that can be used in the green synthesis of AgNPs.

Consequently, the objective of this study is the production and characterization of silver nanoparticles (AgNPs) using green synthesis methods that employ extracts from three plants of the *Arecaceae* family, as well as the evaluation of the minimum inhibitory concentration (MIC) against Gram-positive and Gram-negative bacteria.

Material and methods

Obtaining leaves of plants from the *Arecaceae* family

Leaf samples were collected from three different species of plants from the *Arecaceae* family on a private land in Santarém, Pará state, Brazil (2°27'50" S and 54°43'33" W), and then stored in plastic bags until use. The HSTM Herbarium at the Universidade Federal do Oeste do Pará (Brazil) confirmed the identification of the species and a voucher for each sample was deposited at the HSTM Herbarium under the code HSTM14068 (*Euterpe oleracea* Mart.), HSTM14069 (*Oenocarpus bacaba* Mart.), and HSTM14070 (*Mauritia flexuosa* L.f.).

This research was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado (National System for the Management of Genetic Heritage and Associated Traditional Knowledge) (SisGen) under registration number A381540

For the synthesis of the AgNPs, approximately 1.5 g of leaves of each plant were decontaminated with a neutral detergent to remove undesirable particles, washed with running water, and cut into small fragments. These were later placed in glass beakers with ultrapure water whose quantity varied according to the initial mass of the leaves until a final concentration of 0.1 g mL⁻¹ (100 mg mL⁻¹) of the extract was obtained.

Preparation of aqueous extracts and synthesis of AgNPs

The aqueous extracts were obtained by heating the water containing the macerated leaves at a temperature of around 70°C for 3 min. on a heating plate (Quimis®). Afterwards, the solutions were filtered into Erlenmeyer culture flasks and then stored in microtubes protected against UV radiation. Silver nanoparticles were synthesized from three plant extracts, which were identified according to the botanical genus: NPE (*Euterpe oleracea* Mart.), NPO (*Oenocarpus bacaba* Mart.) and NPM (*Mauritia flexuosa* L.f.). The extracts were slowly added to a 1 mmol L⁻¹ AgNO₃ aqueous solution (Sigma-Aldrich® St. Louis, USA) which had been previously prepared. The mixtures were incubated under low light conditions at 75°C for characterization after 2:30 h.

Characterization of AgNPs

UV/Vis spectroscopy

The AgNPs were characterized by spectroscopy in the UV/Vis region with the aid of a Quimis®-Q798U spectrophotometer (Quimis®, Diadema, Brazil). Initially, the reaction of the extracts with the Ag⁺ ions was monitored based on the change in coloration and difference between the absorbance values at 450 nm, and afterwards, the absorption curve was described based on the range of λ between 350-500 nm.

FTIR spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) measurements were conducted on a spectrometer (Bruker-Alpha, Billerica, USA) in the range of λ between 500-4000 cm⁻¹ using potassium bromide tablets (KBr, Sigma - Aldrich® St. Louis, USA) with 1 mL of each colloidal solution of the AgNPs, which had been dried for 3 days in an oven at 40°C.

DLS and surface Zeta potential

The Zetasizer Nano ZS equipment (Malvern, UK) was used to measure the size (mean hydrodynamic diameter), the polydispersity index (PDI), and the surface Zeta potential (particle loading). A total of 100 μ L of each sample of the AgNPs were diluted in 900 μ L of ultrapure water and then deposited in an electrode-containing polystyrene cuvette that was inserted into the apparatus and subjected to 10 runs in triplicate with a spreading angle at 173° and a temperature of 25°C.

Scanning electron microscopy (SEM)

Approximately 200 µL of each sample of AgNPs, without dilution, were placed in Eppendorf tubes and centrifuged for 10 min. at 2268 G-force and then a pellet sample was withdrawn and deposited on the stub. This was allowed to dry for 24 hours at room temperature and then analysed by SEM (model JEM 7001F - JEOL, São Paulo, Brazil).

Transmission electron microscopy (TEM)

From each AgNP solution, 100 µL were separated, which was then mixed with 900 µL of distilled water. From this solution, about 3 µL were dripped onto a formvar/carbon grid that was left drying for 24h at room temperature and then subjected to TEM (model 1011 -JEOL, São Paulo, Brazil) for analysis of particle size and morphology.

Minimum inhibitory concentration (MIC)

Duplicate assays were performed on 96-well plates (Corning®) using different concentrations of AgNPs against the growth of *Escherichia coli* ATCC 25922 (Sigma - St. Louis, USA) and *Staphylococcus aureus* ATCC 29213 (Sigma - St. Louis, USA), according to the standardization of the Clinical Laboratory Standard Institute (CLSI, 2015). The bacterial colonies were grown for 24h on Mueller-Hinton agar (MH) (Difco®, Lebanon, USA), then diluted in sterile saline (NaCl 0.85%, Synth, Diadema, Brazil) and the concentration of bacteria was adjusted on the McFarland 0.5 scale, corresponding to 1.5×10^8 CFU mL⁻¹. Afterwards, bacterial suspensions were diluted in MH broth (MHB) (Difco®, Lebanon, USA) and placed in each well at a concentration of 5.0×10^5 CFU mL. The microplates were incubated for 18h at 37°C and the results were measured by the turbidity in each well, which indicated no bacterial growth. Growth control was only performed with culture medium and bacteria (without AgNPs). Negative control was performed by replacing extract samples with Mueller Hinton broth and the positive control was performed with ceftriaxone (Agila, Rio de Janeiro, Brazil) (100 µg mL⁻¹).

Results and discussion

Dynamic light scattering (DLS) and surface Zeta potential analysis

Due to the Brownian motion, the light scattered in the AgNP solution undergoes oscillations over time (Michaelides, 2015). Therefore, the DLS technique was employed to evaluate the differences in particle size in aqueous media and the Zeta potential was used to analyze its surface charge in the medium, in each of the syntheses, as can be seen in Table 1.

Table 1. Data obtained by DLS and potential surface Zeta for the AgNPs synthesized using leaves from plants of the *Arecaceae* family*.

Synthesis	HD (nm)	PdI	Zeta Potential (mV)
NPE	130.43 ± 1.61 ^{ab}	0.689 ± 0.13 ^{aa}	-26.97 ± 0.71 ^{aa}
NPO	352.93 ± 38.74 ^{ab}	0.679 ± 0.23 ^{aa}	-17.2 ± 1.11 ^{aa}
NPM	160.73 ± 10.37 ^{ab}	0.523 ± 0.08 ^{aa}	-20.47 ± 1.95 ^{aa}

*Values are represented as the mean ± standard deviation (n = 3). Distinct lowercase letters in the columns for each variable differ from each other by the Tukey test at 5 % of probability. NPE (*Euterpe oleracea* Mart.), NPO (*Oenocarpus bacaba* Mart.), NPM (*Mauritia flexuosa* L.f.). HD – hidrodinamic diameter. PdI – polydispersity index.

It can be observed that NPE and NPM showed close hydrodynamic diameter (HD), which indicates populations of particles with similar size in the solution; however, the high polydispersity index (PdI) value may indicate a lower level of homogeneity of the AgNPs. Zaheer (2018) used palm oil to synthesize AgNPs and reached diameters of between 100-200 nm. As observed in the table above, the NPO extract presented high HD and PdI indices, which are characteristic of a distinct particle size distribution, in addition to a high polydispersity. Zeta potential values indicate that all samples showed a negative charge in an aqueous medium. This measurement is made according to the presence of ionizable or adsorbed groups on the surface of the particles and correlates with the electrostatic repulsion between the different charges present in the system and consequent stability (Moghaddam, Dadanlou, Khajeh, Rakhshanipour, & Shameli, 2014). According to Gengan, Anand, Phulukdaree, and Chuturgoon (2013), a Zeta potential higher than +30 mV or less than -30 mV indicates a stable system. Furthermore, according to Hanaor, Michelazzi, Leonelli, and Sorrell (2014), the closer the Zeta potential value is to 0 mV, the greater the chance of agglomeration of the particles.

UV/Vis analysis

Initially, the reduction of Ag^+ ions to Ag^0 mediated by leaf extracts was monitored by the color change in the solutions, which was easily identified by the appearance of a yellow color; characteristic of colloidal silver. This phenomenon is related to the surface plasmon resonance (RPS) that transforms the reaction mixture.

There are several papers in the literature that preliminarily analyze the formation of AgNPs using the UV/Vis technique. Caroling, Tiwari, Ranjitham, and Suja (2013) suggest that metallic silver exhibits absorbance characteristics around specific wavelengths. In our study, after 90 min. of reaction, the maximum absorbance was found to be in the range of 420–430 nm (Figure 1), which was similar to that found in other studies that reported the synthesis of AgNPs using extracts from other plants of the Arecaceae family, such as *Elaeis guineenses* (maximum at 428 nm) (Velmurugan et al., 2011), *Hyphaene thebaica* (maximum at 430 nm) (Bello et al., 2017) and *Cocos nucifera* (maximum ranging from 414 to 433 nm) (Zamiri et al., 2011; Roopan et al., 2013; Mariselvam et al., 2014).

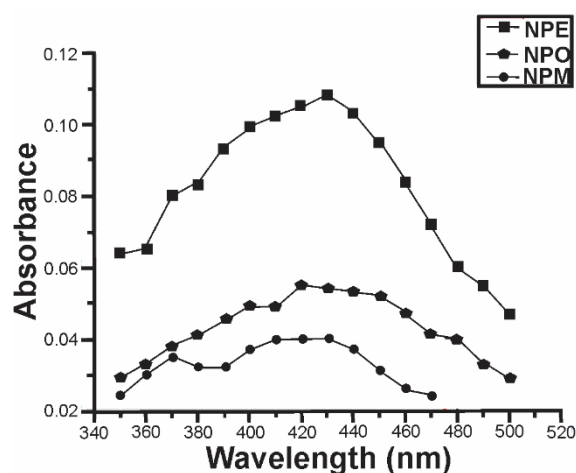


Figure 1. UV/Vis spectra of the AgNPs synthesized by plant extracts from plants of the Arecaceae family. NPE (*Euterpe oleracea* Mart.). NPO (*Oenocarpus bacaba* Mart.). NPM (*Mauritia flexuosa* L.f.).

The AgNPs synthesized in this study showed spectra with maximum absorbance ranging from 400 nm to 450 nm. According to Shankar, Ahmad, and Sastry (2003) and Mariselvam et al. (2014), the presence of a single band of resonance corresponds to spherical nanoparticles according to Mie's theory and these may be promising for use in antibacterial applications, among others (Huang et al., 2007).

Fourier transform infrared spectroscopy analysis

The secondary vegetable metabolites involved in the synthesis of AgNPs can act as stabilizers of the particles in the solution, as well as in the control of the formation of them, playing a double role in the process of obtaining this nanomaterial (Ahmad, Tay, Shameli, Hussein, & Lim, 2011; Tran, Vu, & Nguyen, 2013).

The analysis by Fourier transform Infrared Spectroscopy (FTIR) provides information about the functional groups present in the secondary metabolites of plants that may be responsible for the reduction of Ag and stabilization of the formed AgNPs. The results of the analysis by FTIR were very similar in relation to the bands found, which indicates the similar functional groups in the three extracts (Figure 2). The band around 1070 cm^{-1} corresponds to C-O and C-O-C stretches of primary alcohols and phenolic compounds (Mariselvam et al., 2014; Ali et al., 2015; Anandalakshmi, Venugobal, & Ramasamy, 2016; Govarthanan et al., 2016). The intense elongation at 1384.9 cm^{-1} may be due to the C-O bond of alcohols or flavonoid and terpenoid compounds present in the leaf extracts, C-N compounds and C-O-C bond (Philip, 2011; Niraimathi, Sudha, Lavanya, & Brindha, 2013; Anandalakshmi et al., 2016; Ravichandran, Vasanthi, Shalini, Shah, & Harish, 2016). Huang et al. (2007) correlate the appearance of this band with the residual nitrate group (NO_3^-) in the solution. The band around 1633 cm^{-1} is described as vibrations of C=C groups of aromatic compounds, interactions between C=O groups and also compounds that have nitrogen (Khalil, Ismail, El-Baghdady, & Mohamed, 2014; Mariselvam et al., 2014; Ali et al., 2015; Ahmed et al., 2016; Verma, Hasan, & Banik, 2016). The broad and intense band around 3440 cm^{-1} corresponds to O-H bonds of polyphenols and N-H stretches of proteins (Mariselvam et al., 2014; Ahmed & Ikram, 2015; Ali et al., 2015; Ahmed et al., 2016; Bello et al., 2017).

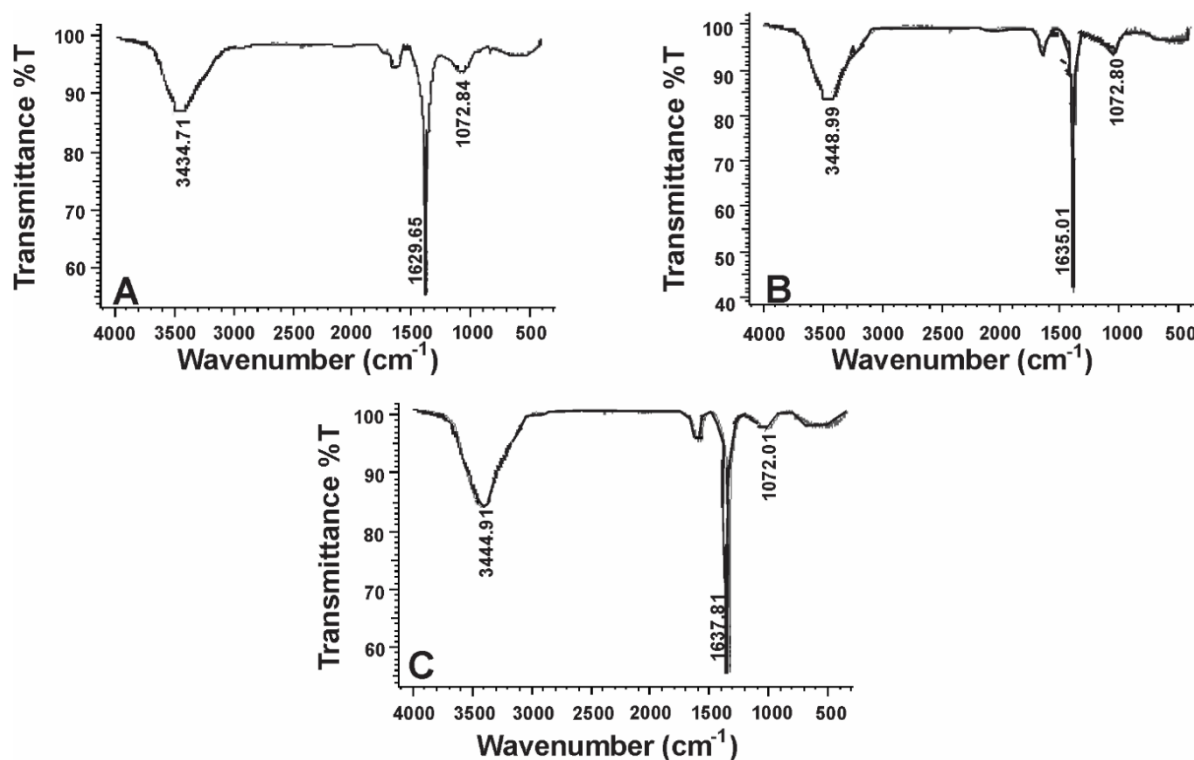


Figure 2. FTIR spectra of AgNPs synthesized from the extracts of different plants from the Arecaceae family. (A) NPE (*Euterpe oleracea* Mart.) (B) NPO (*Oenocarpus bacaba* Mart.) (C) NPM (*Mauritia flexuosa* L.f.).

Even without a phytochemical quantification of the substances in each extract, it was generally observed that the synthesis may have occurred by a mechanism of silver reduction and coating of the AgNPs by the phenolic compounds and proteins adsorbed on their surface, leaving them stable through their redox capacity (Shang et al., 2001; Martínez-Castañón, Niño-Martínez, Martínez-Gutierrez, Martínez-Mendoza, & Ruiz, 2008). Proteins can form a coating that keeps AgNPs free from agglomeration and their association in the synthesis and stabilization of AgNPs from plant extracts has already been reported (Sanghi & Verma, 2009; Nabikhan, Kandasamy, Raj, & Alikunhi, 2010; Zayed, Eisa, & Shabaka, 2012).

Scanning Electron Microscopy

Under Scanning Electron Microscopy (SEM), the morphology of AgNPs was observed as being largely spherical. The presence of some agglomerates that had formed was very visible as white spots, which confirmed the formation of the AgNPs (Figure 3a-c), similar to that previously seen in the literature (Bello et al., 2017). This corroborates the hydrodynamic diameter in suspension, which was obtained by DLS. Some studies also show that the shiny layer on the surface of the nanoparticulate agglomerates which formed may be due to a covering produced by the plant extracts (Zaheer, 2018) and it is interesting to observe the analysis of the main chemical components obtained by FTIR for each extract. These results correlate well with other studies of AgNP synthesis that used plant extracts from the *Arecaceae* family, since under SEM, spherical morphology and also the formation of agglomerates for the nanostructures could be observed (Velmurugan et al., 2011; Roopan et al., 2013).

Transmission electron microscopy

Transmission electron microscopy (TEM) has already been used to characterize the size and shape of nanostructures. In this study, high-resolution TEM images (~0.1-0.2 μm) showed polydispersed AgNPs without the formation of large agglomerates for all the extracts used (Figure 4a-c). Corroborating with the maximum absorption peaks in UV/Vis and RPS band, the predominance in particle morphology was spherical, as observed by Kumari and Philip (2013). Mariselvam et al. (2014) and Bello et al. (2017) showed that the distribution of AgNPs, as well as their morphology in other plants of the *Arecaceae* family, were very similar to the distribution presented here. In a recent study, Zaheer (2018) observed AgNPs of palm oil with a dark layer on the surface and the formation of aggregates. Other studies evidence the aggregation formation of AgNPs using other species of the *Arecaceae* family (Roopan et al. 2013; Govarthanan et al. 2016; Velmurugan et al., 2011; Zaheer, 2018).

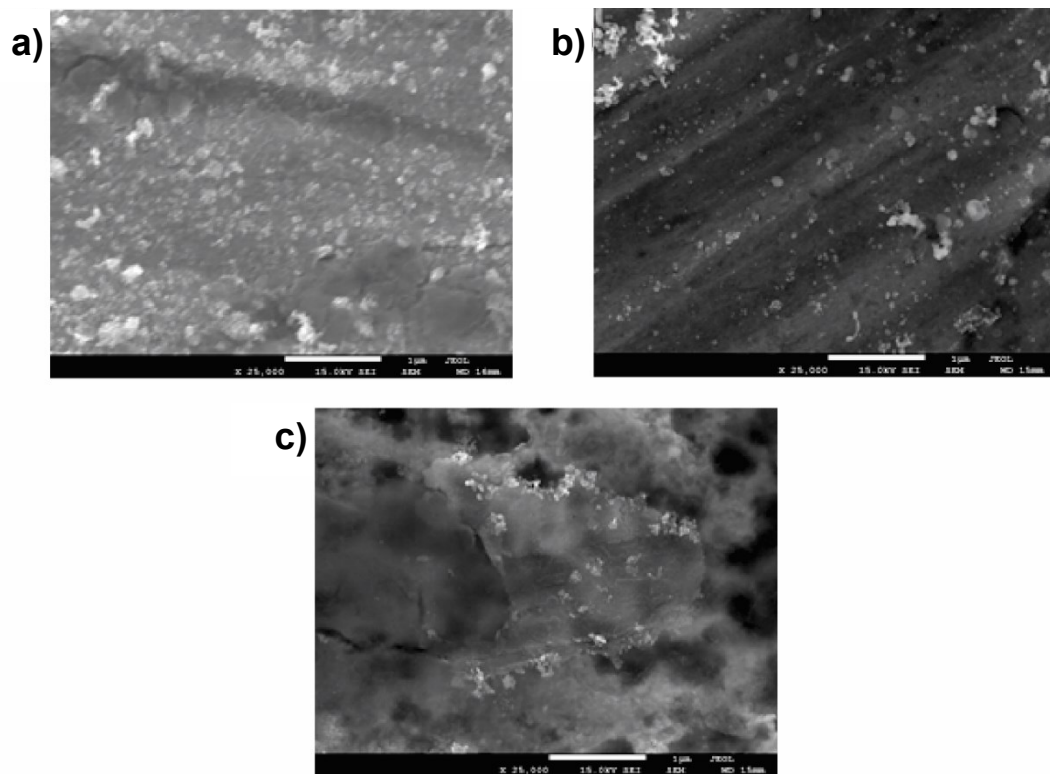


Figure 3. Scanning electron microscopy images of AgNPs synthesized by extracts from plants of the family Arecaceae. (A) NPE (*Euterpe oleracea* Mart.) (B) NPO (*Oenocarpus bacaba* Mart.) (C) NPM (*Mauritia flexuosa* L.f.).

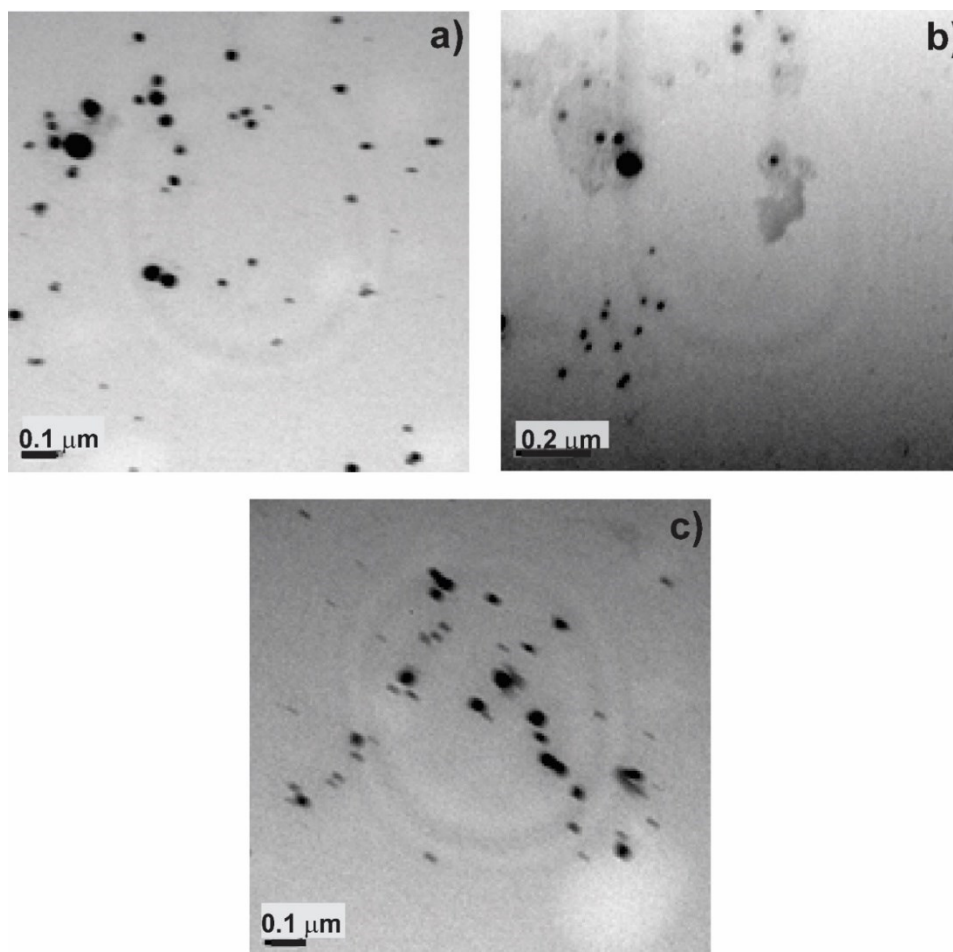


Figure 4. Transmission Electron Microscopy images of AgNPs synthesized by extracts of plants from the Arecaceae family. (A) NPE (*Euterpe oleracea* Mart.) (B) NPO (*Oenocarpus bacaba* Mart.) (C) NPM (*Mauritia flexuosa* L.f.).

Minimum inhibitory concentration

Metallic silver has a well-known antibacterial effect on several microorganisms. In this study, the Minimum Inhibitory Concentration (MIC) of the AgNP samples synthesized using the plant extracts was tested at different concentrations against the bacteria *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 and the result was given by the absence of turbidity in the wells. Table 2 summarizes the values of inhibition of bacterial growth after 24 hours of treatment.

Table 2. The minimum inhibitory concentration of AgNPs tested against bacteria.

	NPE	NPO	NPM
<i>Escherichia coli</i>	5.5 $\mu\text{g mL}^{-1}$	5.5 $\mu\text{g mL}^{-1}$	2.75 $\mu\text{g mL}^{-1}$
<i>Staphylococcus aureus</i>	21.25 $\mu\text{g mL}^{-1}$	21.25 $\mu\text{g mL}^{-1}$	21.25 $\mu\text{g mL}^{-1}$

NPE (*Euterpe oleracea* Mart.) (B) NPO (*Oenocarpus bacaba* Mart.) (C) NPM (*Mauritia flexuosa* L.f.).

A dose-dependent relationship was observed with regards to the concentrations of the AgNPs and their effects on the growth of bacteria. For example, the NPM showed a greater antibacterial effect on *Escherichia coli* and, in general, this microorganism presented greater sensitivity to the lower concentrations tested (Figure 5a). Durán et al. (2010) observed an accumulation of AgNPs in *Escherichia coli* cell membranes and cells and explained that this difference may be related to the size of the particles, since the smaller the size, the larger the area that could interact with the microbial cells. The minimum values found for doses of AgNPs against *Staphylococcus aureus* were identical to those of *Escherichia coli* (Figure 5b).

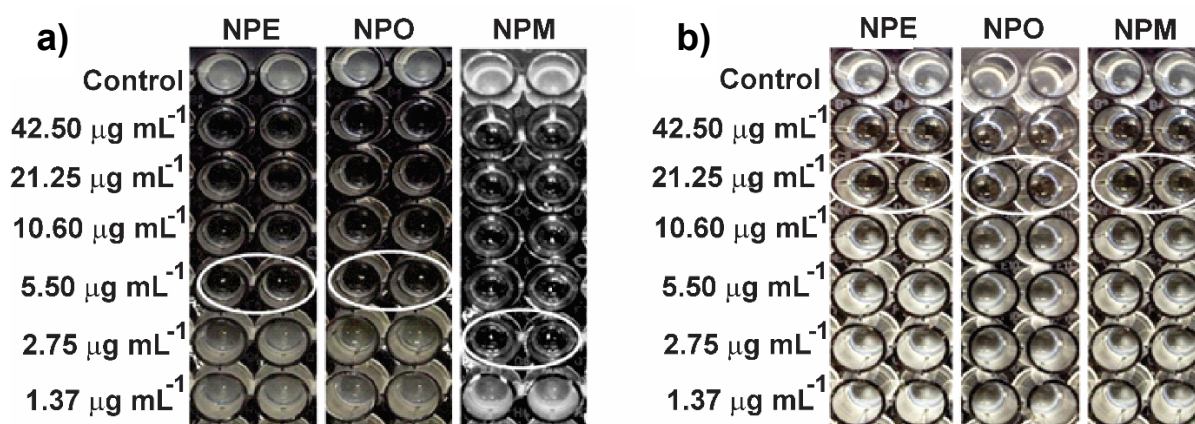


Figure 5. Minimum inhibitory concentration (MIC) assay for AgNPs against (A) *Escherichia coli* and (B) *Staphylococcus aureus*. The absence of turbidity in the wells indicates the level of MIC of the sample (highlighted).

The mechanism by which AgNPs lead to the decrease of bacteria of clinical interest studied here is not yet fully understood, but some aspects can be discussed. Among them is the peptidoglycan layer, which is more rigid and complex in Gram-positive bacteria, such as *Staphylococcus aureus*, and which may prevent the entry of infectious agents into their cells. Furthermore, the electrostatic attraction between the positively charged AgNPs and negatively charged bacterial membrane surface releasing Ag^+ ions into the extracellular medium that act by binding to sulfhydryl (-SH) groups of the enzymes and bacterial DNA alter the normal functioning of microbial respiratory machinery and generate oxidative stress through the formation of reactive oxygen species (ROS) (Nel et al., 2009; Jones & Hoek, 2010; Soenen et al., 2011; Vijayakumar, Priya, Nancy, Noorlidah, & Ahmed, 2013; Zhang, Cheng, Zhang, Xue, & Fu, 2013). Besides this, the natural bioactive compounds present in the chemical composition of the extracts may also influence this biocidal action in regards to bacteria. However, only more in-depth studies of these chemicals can elucidate these points and make AgNPs synthesized via these promising Amazonian plants suitable for clinical applications, among others. Studies with other species of the *Arecaceae* family also found antibacterial action against the same microorganisms studied here, for example, AgNPs from *Hyphaene thebaica* reduced the formation of bacterial colonies by up to 99% after 24 hours of incubation (Bello et al., 2017). Mariselvam et al. (2014) tested AgNPs from *Cocos nucifera* and obtained inhibition of the growth of these same pathogens in several concentrations. The antimicrobial activity observed for AgNPs using aqueous extract of *Phoenix dactylifera* L. in the assays of Zaheer (2018) against microorganisms showed that the zone of growth inhibition increased with increasing concentrations of AgNPs, and exceeded 20 mm when the dose was 50 $\mu\text{g mL}^{-1}$.

Conclusion

AgNPs were synthesized from the reduction of Ag^+ metal ions by bioactive compounds present in the plant extracts of different plants from the *Arecaceae* family. These AgNPs presented properties, such as homogeneity and particle size at the nanoscale, in addition to rapid bioreduction. The bactericidal effect of AgNPs against *E. coli* and *S. aureus* presented the concentration ranges required to eliminate these microorganisms by up to 90%. The obtained information showed the promising potential of native plant species from the Amazon for use in nanobiotechnology is expanded by such studies and adds value to this often underexploited raw material.

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