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Antimicrobial and Toxicological Effects of Phytosynthesized Silver Nanoparticles in Contact with *E-Coli* and *C-Elegans*

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Abstract

The eco-friendly synthesis of silver nanoparticles (AgNPs) is reported from the reduction of an aqueous silver nitrate solution (10 mM) using a natural extract of guava leaves (*Psidium Guajava*). The properties of the synthesized nanoparticles were determined by SEM and UV-vis spectroscopy, showing an average particle size of 31±7 nm, with the presence of the wavelength peak at about 414 nm. Antimicrobial capacity was evaluated using *Escherichia coli* ATCC 25922 through the diffusion disc method, showing inhibition zones up to 1.5 mm with concentration of 3.1 mg/mL of nanoparticles. The toxicity of AgNPs was evaluated using the nematode *Caenorhabditis elegans* as biological model exposing at different AgNPs concentration (500, 1000, 2000 and 4000 μg/L). After 24 h of contact time no significant toxicity effects were observed on mortality, locomotion, and growth, suggesting the potential of these nanoparticles for biological applications.

Keywords: AgNPs, eco-friendly, *Escherichia coli*, toxicity, *C. elegans*

1. Introduction

Silver nanoparticles (AgNPs) have been gaining scientific attention owing to their wide range of applications, such as the production of smart fibers for antimicrobial clothing, design of optical sensors, and generation of color depending fabrics, which depend on particle size and semiconducting properties [1, 2]. The green chemistry has been considered as a promising route to synthesize nanomaterials. In this method, chemical components from plants are used as reducing agents to promote the formation of nanoparticles in aqueous media [3, 4]. In addition, these components can act as capping agents to render colloidal stable nanoparticles suspension [5, 6]. Shakeel Ahmed et al. reported a series of extracts and microorganisms for synthesis of gold nanoparticles such as coriander leaf extract (6.75 - 57.91 nm), *Couroupita Guianensis Aubl* extract (26±11 nm) and *Magnolia kobus* leaf extract (5-300 nm)[7].
Some concerns regarding the implementation of nanoparticles in biomedicine and biotechnology applications are related to the possible toxic effects that can be generated by contact with living organisms and ecosystems[8]. Lokina et al. studied the synthesis of silver nanoparticles for antimicrobial and cytotoxic applications using cervical carcinoma HeLa cell as an *in-vitro* model. From these measurements, it was observed that silver nanoparticles were able to cause cancer cell killing at high concentrations of 1000 μg/mL [9]. Based on the *C. elegans* model, studies with gold nanoparticles have been assessed to evaluate their transport through the intestinal and dermal walls supported on the absence of endocytosis [10]. Moreover, iron nanoparticles have been used for studies with *C. elegans* due to their optical qualities [11], as well as titanium dioxide nanoparticles have been in contact with this model to determine the level of toxicity associated to this nanomaterial [12].

2. Experimental

2.1.Green synthesis of silver nanoparticles (AgNPs)
The extract of guava leaves was carried out by the infusion method, from which the dried leaves were placed with distillated water at 100 ºC and then filtered and concentrated from 1 L to 100 mL. To obtain AgNPs, 5 mL of the extract of guava (*Psidium Guajava*) leaves was added dropwise to 50 mL of 10 mM silver nitrate solution ($AgNO_3$). The reaction was carried out at room temperature for 1 h at 100 rpm, using a magnetic stirrer. Afterwards, the nanoparticles were precipitated by centrifugation at 5000 rpm for 10 min. Finally, AgNPs were suspended in 10 mL of distilled water at pH 10 by adding two drops of sodium hydroxide solution (2 M).

2.2.Antimicrobial capacity
The AgNPs were tested by the Kirby-Bauer disc diffusion assay method to evaluate the antimicrobial activity [13]. The test was performed using *E.coli* ATCC 25922, the bacteria was spread on nutrient agar previously filled into petri

plates using a bacterial wing. The paper discs (pore of 12 μm) were sterilized and then they were wetted with silver nanoparticles solution (3.1, 1.5 and 1 mg/mL) and placed over the agar with the bacteria, then it was incubated at 37 °C for 24 h.

2.3..Toxicological assay

C. elegans were age-synchronized using bleach solution (0.5 M NaOH, 0.8% HClO). The worms were incubated in Petri dishes with K agar at 20 °C, previously seeded with *Escherichia coli* OP50 [14]. The concentrations used were 500, 1000, 2000 and 4000 μg/L of AgNPs, and K medium was used as control.

Locomotion: Nematodes in L2 larval stage were exposed for 24 h to AgNPs concentrations. Then they were scored for the number of body bends in 20 s. A body bend was assumed as a change in the direction of the posterior bulb of the pharynx along the axis [15].

Growth: L2 larval stage were exposed to AgNPs (500, 1000, 2000 and 4000 μg/L) for 72 h, with addition of *Escherichia coli* OP50 each 24 h, each experiment was done by triplicate. The body length after exposure was measured using images from a Nikon smz 745T microscope and the ImageJ software [15].

Mortality: C. elegans in L4 larval stage were exposed for 24 h to AgNPs (500, 1000, 2000 and 4000 μg/L). About 15 ± 1 nematodes were used for this treatment [15].

2.4.Characterization

UV-Vis spectroscopy, using a Labomed, Inc. UV 2650. A scanning electron microscope (SEM) Quanta FEG 650, coupled with an energy-dispersive X-ray spectroscopy (EDAX), was used to determine the size and size distribution of the silver nanoparticles. EDAX measurements were recorded by using an APOLO EDAX X detector with a resolution of 126.1 eV (in. MKα). Backscattered electron images were taken with an accelerating voltage of 20 kV and size spot 5. A Microscope Nikon smz 745T was used to take the image of inhibition zone.

3. Results and Discussion

3.1.*UV-Vis spectroscopy of silver nanoparticles.*

UV-vis absorption spectra were recorded for samples of silver nanoparticles suspended in distilled water (pH 10) and an aqueous sample of the extract of guava (*Psidium Guajava*) leaves. The UV-vis spectrogram is displayed in Figure 1, from which a maximum wavelength peak at 414 nm was observed for silver nanoparticles. This peak can be associated to the phenomenon of surface plasmon resonance (SPR) exhibited by the polarization of the electrons around the nanoparticles when an electromagnetic wave strikes them [5,16]. In addition, no wavelength peak was detected for the sample of extract of leaves in the range from 400 to 500 nm, confirming the successful synthesis of silver nanoparticles. This result is in agreement with the wavelength peak observed by Prakash and coworkers (2013) who reported values ranging from 400 to 450 nm, evaluating

aliquots of silver nanoparticles at different periods during the formation of the nanomaterial. In this research an aqueous extract of *Mimusops elengi* was employed [5]. Similar results were published by Pulikotil et al. (2014) using pine mushroom extract [16].

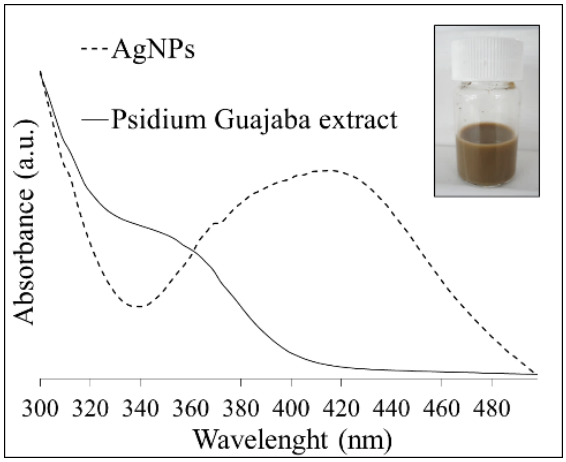

Figure 1. UV-vis spectra for the *Psidium Guajava* leaves extract and the synthesized silver nanoparticles

3.2.Determination of size and size distribution

The SEM image obtained for the synthesized silver nanoparticles is illustrated in in the Figure 2.a. The ImageJ program, provided by NIH, was used to determine the size and size distribution of the nanoparticles (Figure 2.b.). From this measurement, an average size of 31 ± 7 nm was estimated. Additionally, an elemental composition of about 75% weight for the silver atoms present in the sample was determined from EDAX as shown in Figure 2.c. The presence of Na and Cl ions in the sample corresponded to the adjustment of pH in the nanoparticles solution using NaOH and HCl, respectively, to suspend the nanomaterial by electrochemical charge.

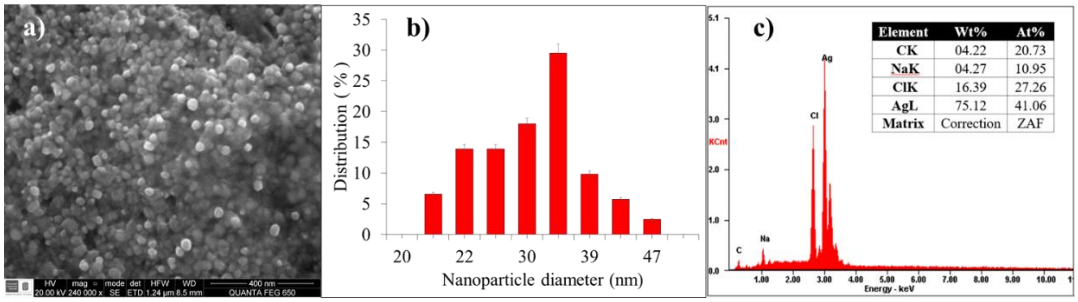

Figure 2. (a) Scanning electron microscopy image at 400 nm, (b) size distribution histogram and (c) EDAX spectrum of the synthetized silver nanoparticles.

3.3.Evaluation of the antibacterial activity of AgNPs.

Antimicrobial activity of silver nanoparticles was evidenced by the effect of silver ions (Ag^+) in contact with a strain of *Escherichia coli* ATCC 25922. The results of antimicrobial activity of the silver nanoparticles are showed in Figure 3, according to Kirby-Bauer's disc diffusion assay method. Antimicrobial activity in each one of the Petri dishes evidenced the presence of inhibition zone diameters after 24 h of exposure time, showing a higher inhibition zone in the paper disc with the highest concentration of the AgNPs. The antimicrobial effect was attributed to the silver cations interacted with the plasmatic membrane of the bacteria causing cell lysis [13]. The electrostatic attraction between the positive superficial charges of the AgNPs and the negative charge of the cellular membranes of the *E.coli* promotes their destruction of the membrane cell causing the antimicrobial effect [17]. Pulikotil et al (2014) studied the antimicrobial effect between the silver nanoparticles and pathogen microorganisms such *B. subtilis* and *E.coli*. The authors reported that the interaction between silver cation and the bacterial membrane caused damage in the intracellular metabolic activity generating the cell death [16].

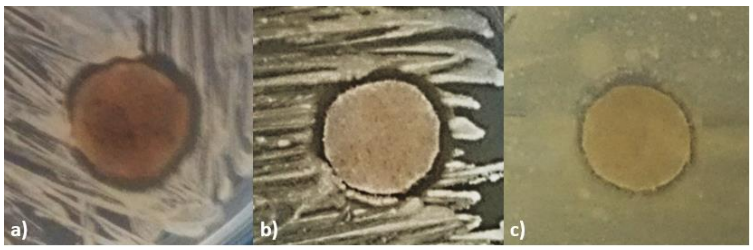

Figure 3. Kirby-Bauer's disc diffusion assay (a. 3.1 mg/mL, b. 1.5 mg/mL and c. 1 mg/mL)

3.4.Toxicological analysis of AgNPs on C. elegans

The results of the locomotion behavior is presented in Figure 4.a. The inhibition in the basic movements occurred at all concentrations examined related to the control ($P < 0.05$). However, there were not significant differences between concentrations. The highest reduction in the body bends (41 %) occurred after exposure to AgNPs at 4000 μg/L in relation to the control group. Effects of AgNPs on locomotion of *C. elegans* have also been observed by Contreras et al. (2014). The movement inhibition has been related to adverse epidermal damage to the cuticle and neurotoxicity [18].

The body length of *C. elegans* after 72 h-exposure is displayed in Figure 4.b. The results showed that growth inhibition presented in the nematodes was concentration-dependent. Higher deviations were observed in the greater concentrations than 282 and 177 μm for 500 and 1000 μg/L, respectively, as contrasted with deviations of 6 and 11 μm for 2000 and 4000 μg/L, respectively. This result can be attributed to the lower concentrations of AgNPs used in the experiment, as the nanomaterial is more dispersed in the K medium solution, affect-

ting in this way the C. elegans to a lesser extent. On the other hand, in a more saturated medium, it was observed that the nematodes were affected by the high concentration of nanoparticles. Hunt et al. (2014) also reported reduction on body length of *C. elegans* after exposure to AgNPs [19].
The mortality results of *C. elegans* exposed to different concentration of AgNPs are presented in Figure 4.c. The mortality was concentration-dependent. Nematodes exposed to 500 and 1000 μg/L of AgNPs showed a little increase in mortality of 1.3 and 3.5% with respect to the control, respectively. In contrast, the concentrations of 2000 and 4000 μg/L showed an increase of 18 and 24.5%, respectively in relation to nematodes not exposed to the nanomaterial. The highest mortality was 57% at 4000 μg/L. Starnes et al. (2015) reported a 78% mortality of the nematodes against a concentration of 4000 μg/L of AgNPs, demonstrating that nanoparticles synthesized with solvents based on plants are less lethal than those generated using toxic solvents [20]. On the other hand, Ellegaard-Jensen et al. (2012) also reported that AgNPs have lethal effects on *C. elegans* [21].

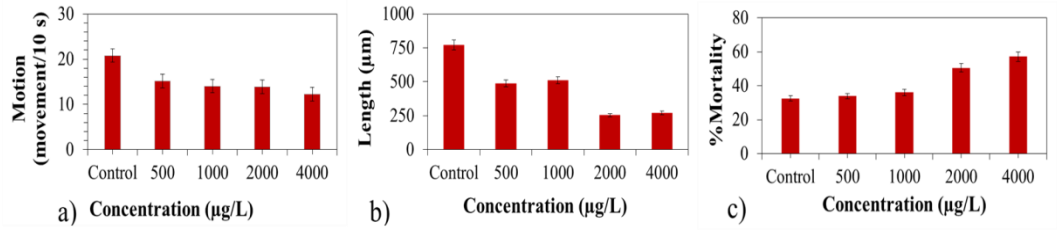

Figure 4. Toxicological assay a) effects of AgNPs on the locomotion of *C. elegans*, b) Body length of *C. elegans* after exposure to AgNPs and c) Mortality on *C. elegans* after exposure to AgNPs.

Conclusions

This research revealed that the extract of guava (*Psidium guajava*) leaves has phytochemical compounds, which work as effective reducing agents in the synthesis of silver nanoparticles, with an economic methodology and environmentally friendly process. This process produced nanoparticle sizes with potential antimicrobial uses in the modification of materials, such as natural fibers and polymers, to be used in the elaboration of food packing materials. In addition, the toxicity of AgNPs on *C. elegans* was concentration-dependent, suggesting their application at low concentrations in order to avoid effects in living organisms.

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