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Punica granatum: A plant with a high potential for the synthesis of silver nanoparticles

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ABSTRACT

Objective: To use pomegranate (*Punica granatum* L.) extracts as a reducing agent for a simple and eco-friendly biosynthesis of silver nanoparticles and to test the latter's antimicrobial potential against different bacteria.

Methodology: The leaves and peel of ripe fruits of pomegranate were used to prepare aqueous and methanolic extracts to synthesize silver nanoparticles (AgNP). Then we evaluated these nanoparticles with techniques that allowed us to define their size, shape, and dispersion quality in aqueous media. To characterize the AgNPs we resorted to UV-Vis spectroscopy and dynamic light scattering (DLS). We determined their antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Staphylococcus aureus*.

Results: UV-Vis spectra were observed with absorption peaks at 425 nm for the nanoparticles synthesized with both aqueous and methanolic extracts. Regarding size, distribution, and dispersion in solution, the zeta potential shows that the surface charge in all cases is negative, with an average value ranging from -28.0 mV to -52.0 mV. Smaller nanoparticles were obtained from the aqueous extracts of the peel and leaves, with values between 1.42 - 2.96 nm, while the methanolic extracts yielded nanoparticles of 10 - 19.04 nm. Inhibition of microorganisms proved to be best against *Escherichia coli*, with a minimum inhibitory concentration (MIC) of 0.83 μ g/mL up to 6.68 μ g/mL.

Conclusions: This study suggests that pomegranate extracts synthesize small-sized, well-dispersed silver nanoparticles with an almost spherical morphology, and good antimicrobial activity against gram-negative bacteria such as *Escherichia coli*.

Keywords: *Punica granatum*, Nanoparticles, Green synthesis, Antimicrobial properties.

INTRODUCTION

Pomegranate (*Punica granatum* L.) originated in Central Asia. Its introduction in America dates to approximately 1500 - 1600. For centuries it has been cultivated for its fruit in warm regions of the world. It is a deciduous shrub or tree that grows in temperate and cold regions. Its fruit is edible, round-shaped, and usually pink-to-reddish-colored. It

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is famous for its beneficial properties for health, and is therefore a functional food, mainly because it is rich in polyphenols. In addition, its peels, leaves, and flowers are used to prepare different meals [1–8].

The ecological synthesis of silver nanoparticles has gained popularity because of its inexpensive and environmentally friendly approach. The green synthesis of nanoparticles uses different reducing agents, among which fruit extracts stand out for being innovative, ecological, profitable, and well preserved when synthesized [7, 9, 10]. Nanotechnology is a constantly evolving discipline with practical applications in various fields, which makes it appealing for industries such as food, cosmetics, construction, textiles, electronics, energy, and the health sector [6, 11, 12]. Metal-based nanoparticles ranging from 1 to 100 nm show good physicochemical properties, especially silver nanoparticles, which have chemical stability, good conductivity, and antimicrobial, antiviral, antifungal, and cytotoxic activity [6, 10]. They are particularly suitable as antimicrobials since bacteria do not have an adaptation strategy that can achieve universal resistance to all metals. Thus, if an antimicrobial metal fails, other metal-based antibiotics can intervene. This allows for research opportunities revolving around the properties of metallic nanomaterials to produce effective varieties of antimicrobial agents [13–16].

Knowledge about nanomaterials from biocompatible agents such as plants is relevant for broader applications. For this reason, our objective was to develop silver nanoparticles from pomegranate extracts to test their antimicrobial potential against gram-positive and gram-negative microorganisms.

MATERIALS AND METHODS

Vegetal material

We evaluated ripe fruits and leaves of pomegranate (*Punica granatum* L.) using the fruits' epicarp. First, we cut down the epicarp and let it dry in the shade at room temperature (25 ± 2 °C) until a constant weight was obtained. It was then reduced to fine powder using a mechanical mill and sieved through a no. 44 mesh. The same process was conducted with the leaves. The powders were stored at room temperature, and protected from light and humidity for subsequent experimental use.

Extract preparation

Aqueous extract

We prepared aqueous extracts by infusing the leaves and the epicarp. For this purpose, we used the fine powder previously obtained and distilled water brought to a boil (1:10 ratio). We removed the infusion from the heat and kept it covered for 10 min. Afterwards, we used Whatman paper grade 40 for filtering. We kept the filtrates protected from light for subsequent use. Each aqueous extract destined for evaluation was prepared at the time of each test.

Methanolic extract

We used 100 g of each materials' fine powder. Following Patra, Dhal, and Thatoi (2011), we macerated the vegetal powder in methanol for five days to obtain the polar compounds

[17]. Then, using a rotary evaporator (BUCHI R-124), methanol was evaporated and the dry extract retrieved.

Green synthesis of silver nanoparticles (AgNP)

We conducted a green synthesis of silver nanoparticles according to the procedures described by Rodríguez-Luis *et al.* (2016) [18]. We used an aqueous AgNO_3 solution (1.7×10^{-4} g/mL) to synthesize the AgNPs from the pomegranate extracts, adding 10 mL of each extract separately to the AgNO_3 solution via magnetic stirring. Once the extracts were added, we adjusted the pH value of the solution to 10 using an NH_4OH solution (Figure 1).

Physical characterization of the AgNPs

To characterize the AgNPs, we used spectroscopy and dynamic light scattering (DLS) according to the procedures described by Martínez-Castañón *et al.* (2008) and Espinosa-Cristobal *et al.* (2013) [13, 18]. We obtained UV-Vis absorption spectra with an S2000 UV-Vis spectrometer (OceanOptics, Inc.). The position of the plasmonic absorption peak is a surface phenomenon characteristic of the metals' material, morphology, and size at the nanometer scale. Silver nanoparticles absorb energy from the electromagnetic spectrum in the UV-Visible range between 400 and 450 nm. All data were obtained thrice for each AgNP. The hydrodynamic radius and the zeta potential were determined by dynamic light scattering using a Nanosizer DLS.

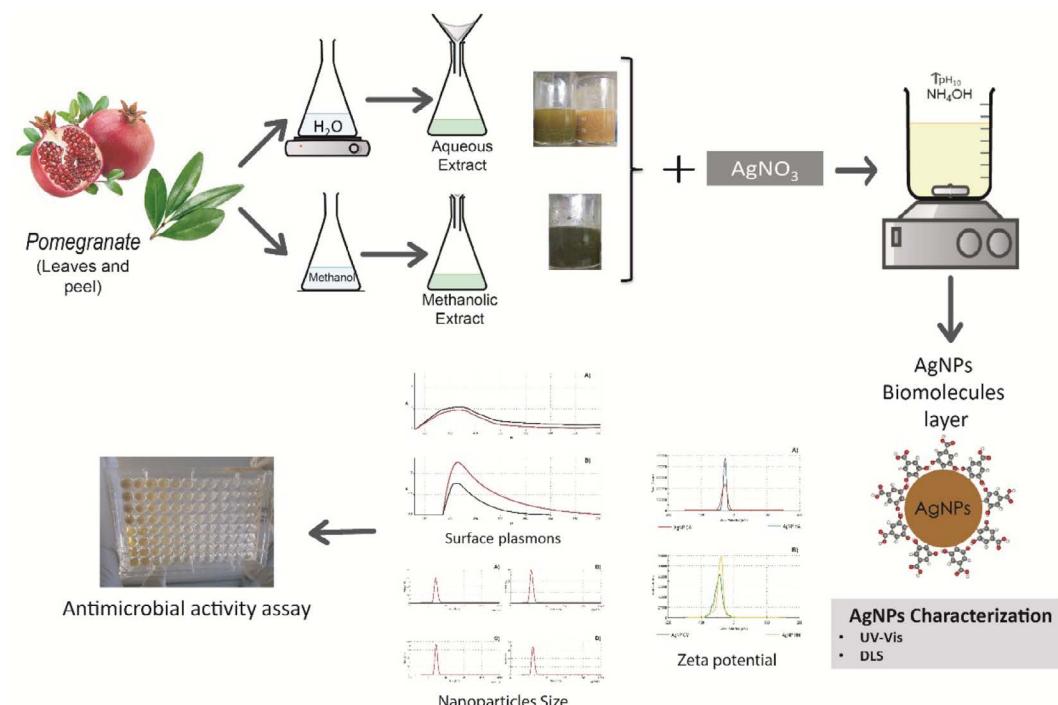


Figure 1. Graphic summary of the AgNPs synthesis process and their physical characterization.

Evaluation of the *in vitro* antimicrobial effect of AgNPs

Selected microorganisms

We resorted to American Type Culture Collection (ATCC) strains, which guarantee the quality of the chosen method. We selected microorganisms that can cause infections in different body parts such as lungs, intestinal tract, skin, or oral cavity. Both gram-positive and gram-negative microorganisms were considered. Among the gram-negative microorganisms, we chose *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Enterococcus faecalis* (ATCC 29212); among the gram-positive, *Staphylococcus aureus* (ATCC 29213) and *Streptococcus mutans* (ATCC 25175).

Standard Microdilution Method (MIC)

The antimicrobial activity of AgNPs was tested using the standard microdilution method that determines the minimum inhibitory concentration (MIC) leading to the inhibition of bacterial growth. For the assays, we followed the procedure described by Dealba-Montero *et al.* (2017) [20]. We used disposable microtiter plates with 96 wells and serial half dilutions of Mueller Hinton (MH) broth with microorganisms at a 105 CFU/mL concentration. We had two controls: the nanoparticles with no broth or bacterial suspension (SB) were added in the first line of wells, while only MH broth was added in the twelfth line. Lines two to eleven contained MH broth, AgNPs from each extract, and SB—all in serial dilution. The results were read after 24 h of incubation at 37 °C as the MIC of the tested substance to inhibit the growth of the bacterial strain. We extracted a culture from the well where no growth inhibition was observed to conduct a disk diffusion test with MH agar in plates previously labeled with the corresponding concentration. The incubation lasted 24 h at 37±2 °C. We took the results from the plates where the antimicrobial thoroughly eliminated bacterial growth. We tested for methanol to rule out any effect of said substance.

RESULTS AND DISCUSSION

Physical Characterization of AgNPs

We observed UV-Vis spectra with absorption peaks at 425 nm for the nanoparticles synthesized with the aqueous extract of epicarp (AgNP-CA) and for those synthesized with the aqueous extract of leaves (AgNP-HA) (Figure 1.) As for the nanoparticles synthesized with the methanolic extract of both epicarp (AgNP-CM) and leaves (AgNP-HM), absorption peaks appeared at 420 nm in the UV-Vis spectra.

In the absorption spectrum, the position of the absorption peak is associated with the size of the particle. Absorptions lower than 435 nm indicate the presence of small particles of around 3 nm. These results confirm the morphology of nanoparticles, since they comply with the absorption associated with small spherical nanoparticles (between 420 and 450 nm as described by [13]).

Regarding size distribution and dispersion in solution, we obtained the zeta potential for the synthesized AgNPs. As we can see, the surface charge in all cases is negative, with an average value of -28.0 mV for AgNP-CA, -28.7 mV for AgNP-HA, -42.3 mV for AgNP-CM, and -51.3 mV for AgNP-HM (Table 1).

In every stance, the surface charge values represent a state of stability that prevents the nanoparticles from coalescing and from subsequent aggregation. As for the size, the smaller AgNPs came from the methanol extracts and presented values of 1.42 for AgNP-CM and 1.72 ± 0.01 nm for AgNP-HM. In all cases, values may represent the obtention of more homogeneous nanoparticles (Table 1).

Plant extracts have been shown to be rich in polyphenols and other compounds that contain hydroxyl groups, which can reduce silver ions to silver [20, 21]. The surface charge values of the AgNPs indicate that the particles are dispersed or have good dispersion. This characteristic is more notable in particles developed from the methanolic extracts because related molecules of said extracts coat the AgNPs and thus reduce the free energy on the particles' surface, preventing aggregation. This phenomenon is explained in the work of Yang *et al.* (2016) [21], which mentions that the free energy on the surface of nanoparticles—especially those that are being formed—is higher, which renders them unstable and allow them to agglomerate with each other to form larger particles, thus lowering the surface energy. This process is spontaneous; however, in the nanoparticles developed from pomegranate extracts by green synthesis, the phytochemicals adhered to their surface, which helped reduce their free energy and avoid agglomeration.

Evaluation of the *in vitro* antimicrobial effect of AgNPs

To assess the antimicrobial activity of the AgNPs developed in this study, we tested minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for the silver nanoparticles against *E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecalis*. We present the results in Table 2, which shows the AgNPs from both aqueous and methanolic extracts.

Regarding the inhibition of microbial growth by nanoparticles, AgNP-CA and AgNP-HA showed better inhibition against *E. coli*, both with a MIC of $3.38 \mu\text{g mL}^{-1}$. For their part, AgNP-CM presented a MIC of $6.68 \mu\text{g mL}^{-1}$, while AgNP-HM had the lowest MIC with $0.83 \mu\text{g mL}^{-1}$.

By observing the AgNP sizes, we can state that the smallest nanoparticles correspond to the lowest inhibition values. The MIC of all AgNPs is lower when tested against *E. coli*

Table 1. Particle size values for AgNPs obtained from aqueous and methanolic extracts of pomegranate epicarp and leaves.

	Size (nm)	Surface charge (mV)
AgNP-CA	2.89 ± 0.01	-28.7
AgNP-CM	1.42 ± 0.00	-42.3
AgNP-HA	2.96 ± 0.01	-28.0
AgNP-HM	1.72 ± 0.01	-51.3

AgNP-CA represents nanoparticles from the aqueous extract of the pomegranate epicarp, AgNP-CM stands for nanoparticles from the methanolic extract of the epicarp, AgNP-HA are nanoparticles from the aqueous extract of the pomegranate leaves, and AgNP-HM come from the macerated methanolic extract of the leaves. The values are the mean of three repetitions \pm their standard deviation (SD); the value of SD in the surface charge is zero.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for AgNPs from pomegranate aqueous and methanolic extracts.

	CMI ($\mu\text{g mL}^{-1}$)				CMB ($\mu\text{g mL}^{-1}$)			
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. faecalis</i>
AgNP-CA	3.38	6.68	3.38	26.75	6.68	13.37	6.67	26.75
AgNP-CM	6.68	13.37	26.75	53.50	6.68	26.75	53.50	53.50
AgNP-HA	3.38	13.37	6.67	13.37	3.38	26.75	13.37	26.75
AgNP-HM	0.83	1.67	1.67	3.38	0.83	1.67	3.38	3.38

AgNP-CA represents nanoparticles from the aqueous extract of the pomegranate epicarp, AgNP-CM from the methanolic extract of the epicarp, AgNP-HA from the aqueous extract of the leaves, and AgNP-HM from the macerated methanolic extract of the leaves. The values are the mean of three repetitions with a standard deviation equaling zero.

and *P. aeruginosa* (gram-negative microorganisms) than when tested against other bacteria. These results may be due to the cell wall structure of each strain, since gram-negative strains can have thinner cell walls than gram-positive ones. Moreover, the nanoparticles interact with the charge of cell walls and affect permeability [13, 22].

The size of nanoparticles affects their antimicrobial effect. Particles of small dimensions within the nanometric scale were found in all AgNPs obtained from the pomegranate extracts. We know that small AgNPs and the phytochemicals adhered to their surface may have a better interaction with bacteria, which is reflected in their high inhibition activity on some bacterial membranes [13, 16, 20].

CONCLUSIONS

The nanoparticles synthesized from *P. granatum* extracts were of small size, showed good dispersion, and an almost spherical morphology, with a good antimicrobial activity against gram-negative bacteria such as *E. coli*. Therefore, pomegranate could be a source of reducing agents for synthesizing silver nanoparticles. Exploring new natural extracts that allow the development of nanomaterials is essential.

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