

Article

Green Synthesis and Antibacterial Activity of Silver Nanoparticles Obtained from *Moringa oleifera* Seed Cake

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Abstract: In the present work, we report a simple, cost-efficient, and eco-friendly green method to synthesize silver nanoparticles with antimicrobial activity. An ethanolic extract from *Moringa oleifera* seed residue was used as a reducing and stabilizing agent in an aqueous solution of silver nitrate. The synthesized silver nanoparticles' hydrodynamic radius, polydispersity index, and zeta-potential were evaluated by Dynamic Light Scattering. Scanning Electron Microscopy was employed to confirm the size and morphology of the nanoparticles. Synthesis of spherical particles with 127 ± 24 nm was confirmed. After sintering, the product of the synthesis was analyzed by X-ray diffraction. The X-ray diffraction pattern attributed to reflections of the (111), (200), (220), and (311) planes, which are characteristic of silver nanoparticles, confirms the successful synthesis of crystalline face-centered cubic nanoparticles. The antimicrobial activity of the bionanoparticles was tested against *Escherichia coli* BL21(DE3) cells and compared with the effect of a *Moringa oleifera* seed cake extract. Herein, we show that the growth of *Escherichia coli* is significantly affected by the addition of the synthesized bionanoparticles. Addition of the bionanoparticles inhibited the growth and lengthened the lag phase of the bacterial culture.

Keywords: *Moringa oleifera* seed cake; by-products valorization; green synthesis; silver bionanoparticles; antimicrobial activity



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1. Introduction

Given the emergent advances of nanotechnology, the demand for more environmentally friendly processes in nanoparticle (NP) synthesis has risen. Several green synthesis methods have gained great attention in the last years, recurring to non-toxic, biocompatible, and biodegradable reagents [1–3]. The biological synthesis process is simple, does not require any special equipment, and is inexpensive and easily scalable. Although there is still a debate regarding the mechanism and the nature of the molecules responsible for the reduction of metal ions in nanoparticles, the generally accepted green synthesis process involves reduction mediated by a biomolecule; nucleation of the metal atoms; and consequent nanoparticles growth and stabilization.

Different plant extracts have been used in the biosynthesis of nanoparticles, specifically with gold, copper, zinc, iron, or silver. They contain proteins, amino acids, lipids, and phenolic compounds, among other phytochemicals that mediate the bio-reduction of metal ions in the synthesis of NPs and their stabilization. The size, shape, and morphology of the NPs depend on the nature of the biomolecules of the plant extract [4,5].

Several parts of *Moringa (M.) oleifera* plants, such as leaves, flowers, and stem barks, have been extensively used in the synthesis of different types of nanoparticles [6–9]. The plant is native to India and nowadays widespread in southern Africa, South America, and some Asian countries. It is known for its nutritive properties and multiple applications in food, cosmetics, pharmaceutical industries, and others, such as bioremediation (water purification) [10–12]. Parts of the plant are commonly used in traditional medicine [13]. It also has ecological value due to its drought resistance and pest-repelling properties. Additionally, *M. oleifera* has antimicrobial and antioxidant activities.

Many properties have been attributed to the seeds of the plant, namely, antimicrobial, antioxidant, anticancer, and anti-inflammatory, to name a few. However, the seeds of the plant are mostly used as animal nourishment or as biofertilizers due to their nutritional content (they are rich in proteins, lipids, carbohydrates, and important minerals) [14–16]. Shebek et al. showed that a cationic protein (MOCP) from the seeds was responsible for membrane fusion of pathogens, contributing to the *M. oleifera* antimicrobial activity against *Escherichia (E.) coli* [17].

In this work we used an *M. oleifera* seed by-product from the oil extraction industry for the green synthesis of silver nanoparticles (AgNPs). When the oil is extracted from the seeds, a solid residue, the cake (also designated by seed meal), with no apparent commercial value and usually discarded, is left out of the supply chain (Figure 1). Due to the high content of bioactive compounds in the cake, it has a high biotechnological potential with several applications, such as insecticidal, antimicrobial, or flocculating agent [14–16]. Alternative applications would add value to this by-product, thus contributing to a circular economy.



Figure 1. *M. oleifera* seeds (left) and seed cake (right).

Synthesis of AgNPs by chemical, physical, and biological methods has been extensively studied due to their cytotoxic, genotoxic, and catalytic activities, with great impact on medicine and agriculture [7,18–20]. The final size and shape of the obtained AgNPs highly depend on the synthesis conditions, such as the pH of the reaction mixture, temperature, reaction time, concentration of reagents, and electrochemical potential of the metal ion [21–23]. The antimicrobial mechanism by AgNPs is still not completely understood. It is believed that nanoparticles attach to bacterial cell walls and membranes, penetrate inside the cell, and damage intracellular structures and biomolecules (nucleic acids, proteins, and lipids). In the cell, the AgNPs induce the formation of reactive oxygen species that further exacerbate cell damage and disrupt signal transduction pathways, impairing the cell metabolism and leading to apoptosis [24].

Herein, we report the green synthesis and characterization of AgNPs using *M. oleifera* aqueous-ethanolic seed cake extracts (Figure 2). Size, morphology, and crystallinity of the AgNPs were investigated by UV-Visible spectroscopy, Dynamic Light Scattering (DLS), X-ray diffraction (XRD), and Scanning Electron Microscopy-Energy Dispersive X-ray (SEM-EDX). The antibacterial activity of the extract and biosynthesized AgNPs was assessed against *E. coli* cultures in LB broth aqueous medium.

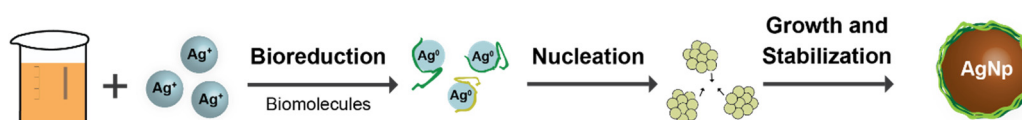


Figure 2. Schematics of the biosynthesis of AgNPs. Biomolecules present in the *M. oleifera* cake extract, such as proteins, flavonoids, or terpenoids, are believed to have an active and essential role in the reduction of Ag ions and consequent nucleation.

2. Materials and Methods

2.1. Preparation of *M. oleifera* Cake Extracts

An extraction was performed using 10 g of *M. oleifera* seed cake (Naturinga, Ferpinta group, Vale de Cambra, Portugal; for composition, see Table S1 on the Supplementary Materials) and 100 mL of a 50% (v/v) aqueous-ethanol solution (absolute ethanol 99.9%, Carlo Erba, Val-de-Reuil, France) at room temperature, with orbital shaking at 200 rpm (Barnstead MaxQ 4000 Orbital Incubator Shaker, Thermo Fisher Scientific, Waltham, MA, USA), for 24 h. The soluble extract was cleared by filtration, first with a laboratory filter paper (43–48 µm) in a Büchner funnel and secondly under vacuum with a membrane filtration unit (0.45 µm cellulose nitrate membrane, Sartorius Stedim Biotech, Göttingen, Germany). The measured pH of the extract was 4.9. The same process was repeated using 5, 15, 20, and 25 g of *M. oleifera* cake.

2.2. Characterization of the Seed Cake Extracts by SDS-PAGE

Protein content of the extracts was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), 12.5% in acrylamide, in reducing and nonreducing conditions, following a standard protocol. A total of 3 µL of a 1:4 dilution of each extract was applied on the gel. Electrophoretic separation was performed at 160 V, 45 mA, for 80 min. Proteins were stained with BlueSafe (NZYTech, Lisbon, Portugal).

2.3. Green Synthesis of AgNPs

The synthesis procedure was adapted from [7]. A total of 10 mL of *M. oleifera* seed cake extracts was added at 1 mL/min flow rate to 40 mL of 1 mM aqueous AgNO₃ (≥99.8%, Sigma-Aldrich, St. Louis, MO, USA), under magnetic stirring and at 80 °C. Qualitative visual confirmation of the formation of AgNPs was possible through color change of the reaction mixture, from light yellow to bronze brown. Major color changes occurred within the first 10 min of reaction at 80 °C, after which the suspension was cooled down to room temperature. After 1 h, no further color changes were observed. A final pH of 5.1 was obtained for the reaction product. The same process was repeated using 5, 15, and 25 g of *M. oleifera* seed cake. For comparison studies, a synthesis control reaction was conducted, replacing the AgNO₃ with Milli-Q grade water.

2.4. Characterization of AgNPs

Biosynthesis of AgNPs was confirmed by UV-Visible spectroscopy (Thermo Fisher Scientific Evolution 201 spectrophotometer, Waltham, MA, USA) using a 1 cm quartz Hellma quartz Suprasil cell (Hellma, Müllheim, Germany).

The hydrodynamic diameter of AgNPs was assessed by Dynamic Light Scattering (DLS) in an SZ-100 Nanoparticle Size Analyzer from Horiba Scientific (Piscataway, NJ, USA), equipped with a 10 mW 532 nm laser and detection at a scattering angle of 90 during 2 min at 25 °C. Before measurements, samples were centrifuged at 10,000× g for 10 min (Sigma 1-14 Sartorius, Göttingen, Germany) at room temperature. The hydrodynamic diameter and polydispersity index (PDI) were determined from triplicate measurements. Analysis of data was carried out using the equipment built-in software, assuming a standard monodisperse form of distribution, a particle refractive index of 1.6 (organic sample), and water settings as dispersion medium (refractive index of 1.333).

The crystalline structure of the bionanoparticles was characterized by X-ray Powder Diffraction (XRD) using a Rigaku MiniFlex II Benchtop X-ray Diffractometer (Neu-Isenburg, Germany), equipped with a Cu-K α line radiation ($\lambda = 1.5406 \text{ \AA}$) in Bragg–Brentano geometry, at the LAQV-Requimte Analytical Laboratory (Caparica, Portugal). The samples consisted of films of synthesized AgNPs (synthesis at 400°C for 3 h, in a Nabertherm muffle furnace with a P320 controller, Lilienthal, Germany), on a flat, low-background silicon samples holder. The crystallite size of the Ag nanoparticles, D , was estimated using the Debye–Scherrer equation [25]:

$$D = \frac{K\lambda}{\beta \cos(\theta)}$$

where K is the Debye–Scherrer constant, a dimensionless shape factor frequently referred to as the crystallite-shape factor (taken as 0.9); λ is the wavelength of the Cu-K α radiation; β is the full width at half-maximum of the (111) plane diffraction peak in radians; and θ is the Bragg angle.

Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) analyses were performed at the Advanced Electron Microscopy, Imaging, and Spectroscopy facility at the International Nanotechnology Laboratory (INL, Braga, Portugal) to evaluate the morphology and elemental composition of the AgNPs. Images were obtained using a Quanta 650 FEG Environmental microscope (Fei Company, Hillsboro, OR, USA) equipped with an Oxford Ultim Max 170 large-area energy-dispersive spectrometer (Oxford Analytical, Bicester, UK). The average diameter of the nanoparticles was calculated using ImageJ software [26].

ATR-FTIR spectroscopy was applied to compare the synthesis reaction products. The spectra were acquired using a Spectrometer Spectrum Two Perkin-Elmer FT-IR (Waltham, MA, USA) equipped with a LiTaO₃ (lithium tantalate) MIR detector with an SNR of 9300:1 and an attenuated total reflection (ATR) diamond cell, in the range of $4000\text{--}400 \text{ cm}^{-1}$.

2.5. Antimicrobial Activity of AgNPs

The AgNPs antimicrobial activity was studied, monitoring the growth inhibition of *E. coli* BL21(DE3) (NZYTech, Lisbon, Portugal) cells harboring the pET-21c(+) vector (Novagen, USA), transformed following the manufacturer's instructions. A transformant was used to inoculate 5 mL of lysogeny broth (LB) medium (NZYTech, Lisbon, Portugal) containing $100 \mu\text{g/mL}$ ampicillin. The culture was grown overnight at 37°C , with orbital shaking at 220 rpm (Barnstead MaxQ 4000 Orbital Incubator Shaker, NH, USA). Three cultures of 100 mL of LB with $100 \mu\text{g/mL}$ ampicillin (in 250 mL flasks) were prepared by transferring 1 mL of the pre-inoculum to 100 mL of LB/ampicillin medium, to which the AgNPs suspension biosynthesized from the 10 g seed cake extract were added at 2.5, 7.5, and 10 mg/mL . The cultures were grown as described above. To ascertain the effect of the *M. oleifera* aqueous-ethanolic extract on the bacterial growth, 10 mg/mL of the 10 g extract was added to a fourth culture and allowed to grow in the same conditions. In parallel and for comparison, an untreated control culture was prepared with no addition of AgNP. All cultures were prepared in duplicates.

Cell growth was monitored measuring the optical density at 600 nm (Ultrospec 10 Cell Density Meter, Biochrom, Holliston, MA, USA) every hour during 9 h. Sigmoid growth curves were analyzed using a modified form of the type I Gompertz model [27]:

$$y = A \exp\left(-\exp\left[\frac{\mu_{\max} e}{A}(\lambda - t) + 1\right]\right)$$

where $\mu_{\max}$ is the maximum specific growth rate (h^{-1}), λ is the lag phase duration (h), A is the upper asymptote (theoretical maximum for the maximum optical density), and t corresponds to the growth time (h).

3. Results and Discussion

3.1. Characterization of Seed Cake Extracts

The protein content of the *M. oleifera* extracts was analyzed by SDS-PAGE, in reducing and nonreducing conditions (Figure 3). In nonreducing conditions (lanes 1 to 4), a major protein band appears with an apparent molecular mass of around 15–16 kDa. Its intensity increases with the amount of seed cake used to prepare the extracts. A second band, with ~6–7 kDa, is also detected on the more concentrated extracts. When the samples were treated with β -mercaptoethanol (lanes 5 to 8), an extra protein band with ~9 kDa was observed, due to the reduction of intersubunits disulfide bridges. Based on the literature, these protein bands are most likely attributed to Mo-CBP₃, a chitin-binding 2S albumin that belongs to the prolamin superfamily of proteins [28]. In the mature form, this protein, with a total molecular mass ~14 kDa, is composed of a small subunit of ~4 kDa and a large subunit of ~8 kDa, covalently bound by several disulfide bridges. Additionally, a mixture of isoenzymes is produced by the *M. oleifera* seeds. The Mo-CBP₃ protein is a compact, thermoresistant (melting temperature of 98 °C), cationic protein, with antifungal activity [29].

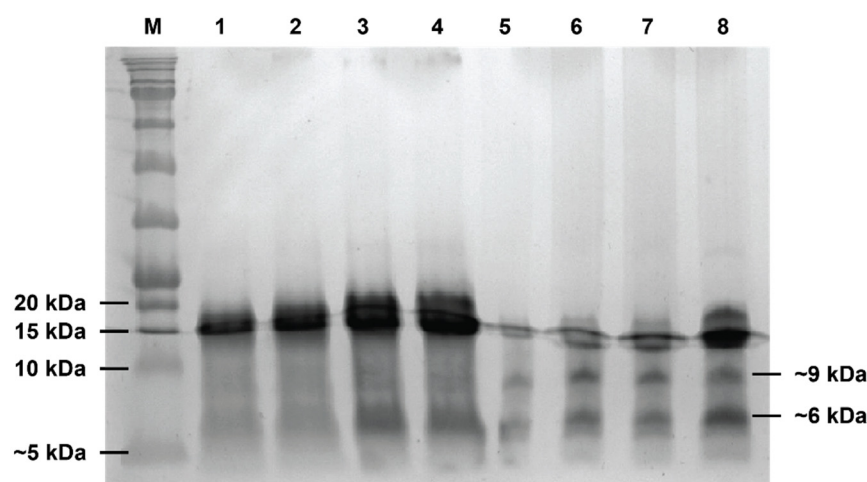


Figure 3. SDS-PAGE analysis of the protein content of *M. oleifera* seed cake extracts by SDS-PAGE. Aqueous-ethanolic extracts from 10, 15, 20, and 25 g of seed cake, in nonreducing conditions (lanes 1 to 4) and treated with β -mercaptoethanol (lanes 5 to 8). A total of 3 μ L of a 1:4 dilution of each sample was applied. M corresponds to the NZYColour Protein Marker I (NZYTech, Lisbon, Portugal).

3.2. Characterization of AgNPs

3.2.1. UV-Visible Spectroscopy

With the addition of the *M. oleifera* extract prepared from 10 g of seed cake (pH = 5.1) to the AgNO₃ aqueous solution, a color change was observed almost immediately, from light yellow to a bronze brown, indicative of the reduction of Ag⁺ ions to Ag⁰ (Figure 4).

After 1 h, the color of the suspension no longer changed, suggesting stabilization of the synthesized bionanoparticles. No visible aggregation/precipitation was detected. A broad absorption band with maximum absorption ~410 nm is present in the UV-Visible spectrum of the final colloidal suspension due to the Ag surface plasmon resonance (SPR) phenomenon (Figure 5) [7,30]. The broadness is justified by the heterogeneity of the NP suspension (see following subsections). The *M. oleifera* seed cake extract per se does not absorb in the same spectral region (Figure 5). When the extract prepared with 5 g of cake was used, no color change of the silver nitrate solution was noticed, suggesting no formation of nanoparticles (see Table S2).

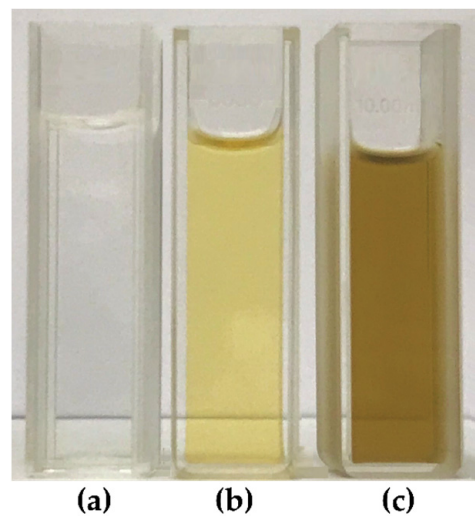


Figure 4. Qualitative monitoring of the biosynthesis of AgNPs. (a) AgNO_3 aqueous solution; (b) *M. oleifera* extract prepared from 10 g of seed cake; (c) biosynthesized AgNPs suspension.

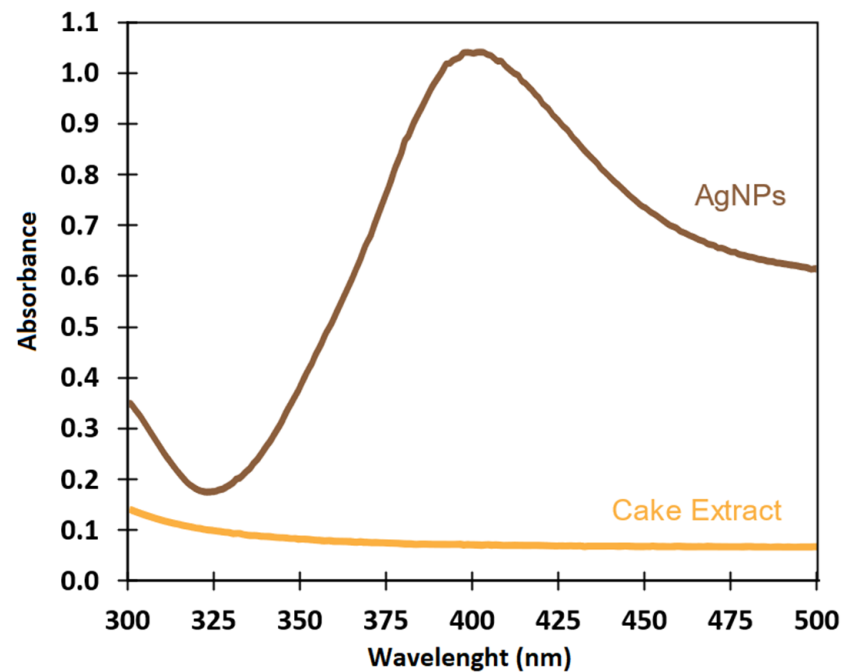


Figure 5. UV-Visible spectra of biosynthesized AgNPs (brown line) and *M. oleifera* seed cake extract (yellow line), both diluted at 10% (v/v).

3.2.2. DLS Spectroscopy

The hydrodynamic diameter of AgNPs was determined by DLS. The biosynthesized nanoparticles were slightly polydisperse ($\text{PDI} = 0.8 \pm 0.1$), with an average hydrodynamic diameter of 174 ± 35 nm (Figure 6 and Table S2). The zeta potential (ζ), or net surface charge, of the bionanoparticles was measured using the Doppler shifting method. The negative zeta potential, -24.1 mV, of the AgNPs indicates predominance of interparticle repulsive forces, which hinders the aggregation of the particles [21]. The zeta potential value, thus, denotes that the biosynthesized AgNPs present a good stability, most likely due to the presence of negative groups at the nanoparticle surface from a biological capping agent.

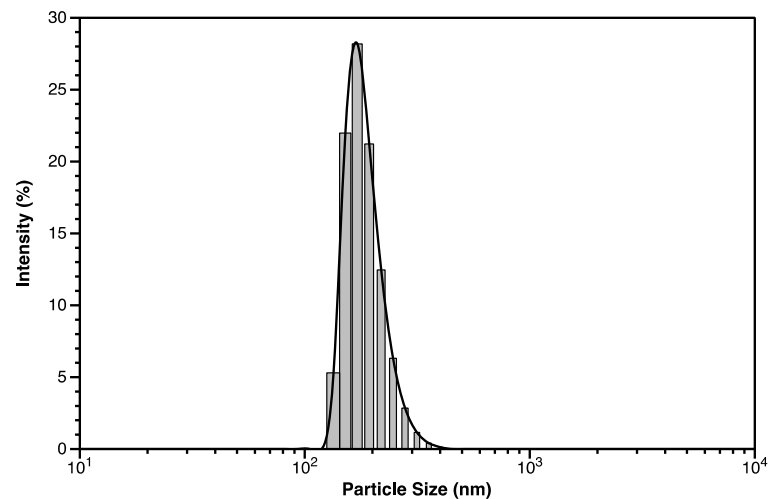


Figure 6. Particle size distribution of synthesized AgNPs synthesized from the 10 g seed cake extract analyzed by DLS at room temperature.

3.2.3. XRD

The structure and crystallinity of the synthesized AgNPs were assessed by XRD (Figure 7). Intense diffraction peaks were observed at 2θ values equal to 38° , 44° , 64° , and 77° , associated with, respectively, (111), (200), (220), and (311) crystal planes, confirming the crystallinity and purity of the AgNPs synthesized. These crystal planes are consistent with face-centered cubic (FCC) nanocrystallites, in accordance with what is described in the literature for this type of nanoparticle [7,31]. The unassigned peaks at around 30° are most likely due to the presence of bio-organic components associated with the AgNPs [32].

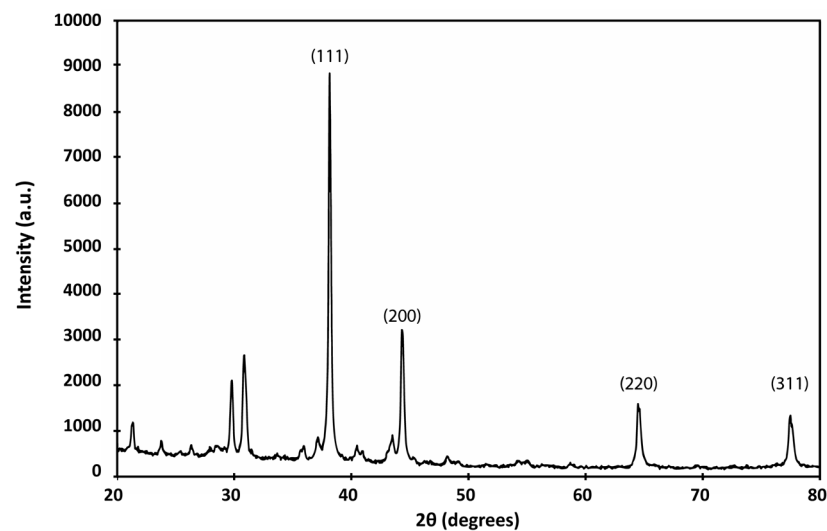


Figure 7. XRD pattern of synthesized AgNPs at room temperature synthesized from the 10 g seed cake extract.

The crystallite size, determined from the (111) plane, was estimated to be 56 nm. As expected, this value is smaller than the size of the particles observed in SEM images (Figure 8), since the Debye Scherer's equation (see Section 2.4) determines the crystalline domain of a single AgNP, not taking into account the aggregation of particles and/or the biological coating molecules.

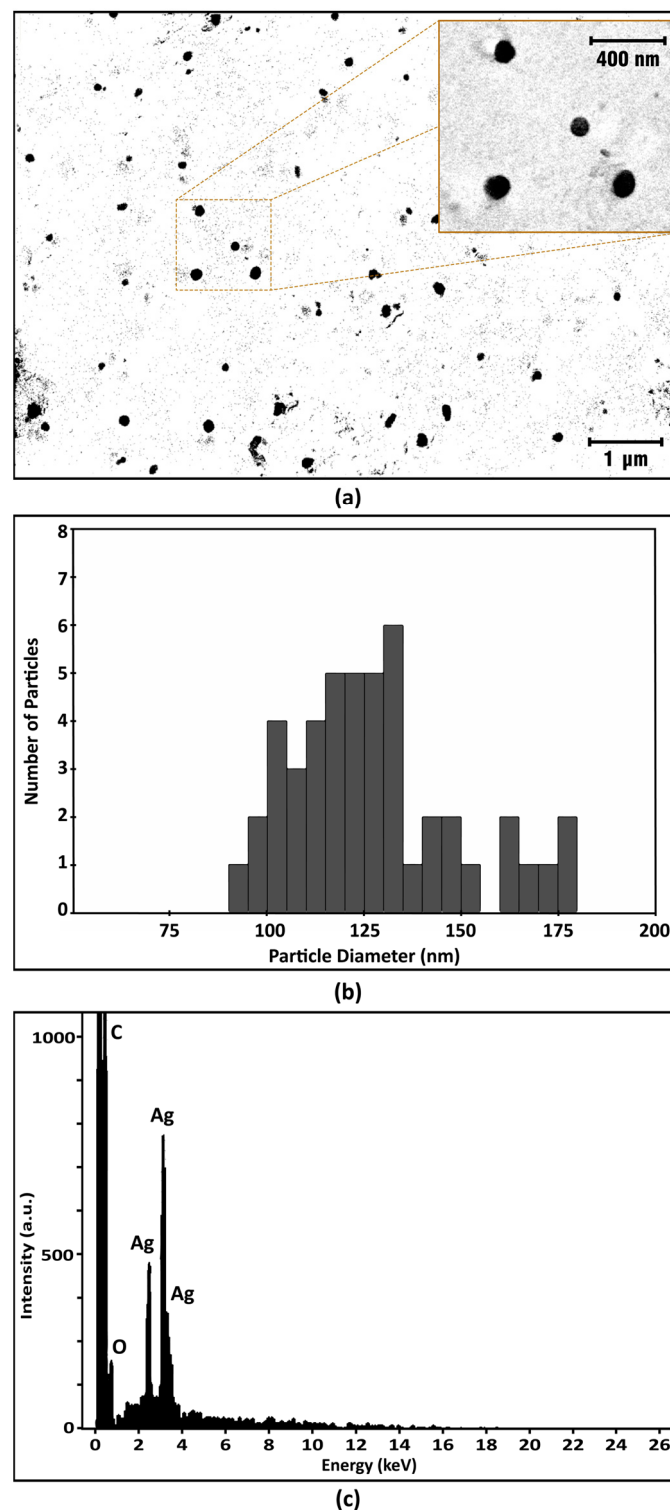


Figure 8. SEM image of synthesized AgNPs (a); size distribution of AgNP observed in the SEM image in (a) with an average diameter of 127 ± 24 nm (out of 47 counts) (b); and SEM-EDX spectrum of AgNPs (c).

3.2.4. SEM-EDX

SEM-EDX was used to assess the shape, size, and elemental composition of the AgNPs biosynthesized using *M. oleifera* seed cake extracts. The spherical shape of the AgNPs was confirmed (Figure 8a). The average size of the nanoparticles was 127 ± 24 nm, with diameters ranging from 90 nm to 180 nm (Figure 8b). This average value is smaller

than the one determined by DLS, since the latter estimates the hydrodynamic size of the nanomaterial. The elemental content of the particles was assessed using EDX (Figure 8c). Intense peaks were detected between 3 and 3.5 keV, confirming the presence of AgNPs [33]. The presence of carbon and oxygen diffraction peaks suggests the successful capping of the AgNPs with biological organic components.

3.2.5. ATR-FTIR Spectroscopy

ATR-FTIR was used to characterize both AgNPs and synthesis control reaction products (Figure 9a,b). The spectra show a broad absorption peak around 3300 cm^{-1} that can be attributed to stretching vibrations of O-H bonds present in phenolic compounds and alcohols, as well as to N-H amide bond stretch. Absorption peaks between 3000 and 2900 cm^{-1} are due to symmetric and asymmetric stretching of C-H bond in methylene groups. Absorption peaks between 1450 and 1000 cm^{-1} result from O-H bending of phenol and carboxylic acid groups, C-N stretching of aliphatic and aromatic amines, and C-O stretches. An important region of the spectra (1800 – 1600 cm^{-1}) is the so-called amide I region that presents absorption bands due to coupling of C=O stretching to in-plane N-H bending and C-N stretching modes of amide groups. Due to the chemical nature of the peptide bond, this region can be used to characterize the secondary structure of proteins with bands centered at specific wavenumbers corresponding to different secondary structure elements. The insets of Figure 9 show the normalized spectral deconvolution based on a Gaussian band-fitting model with six different species (see also Table S3). Bands centered at 1670 cm^{-1} , 1629 cm^{-1} , and 1612 cm^{-1} are usually attributed to β -sheets, while bands centered at 1659 cm^{-1} , 1648 cm^{-1} , and 1640 cm^{-1} can be associated with α -helix and random coil structures. Using the fractional areas of each Gaussian, it is possible to verify that, in the synthesis control reaction products, there is a prevalence of β -sheet elements (ca. 64%), which are halved when the silver nanoparticles are being synthesized (ca. 29%). In the latter case, ca. 59% of secondary structure elements can be attributed to α -helix structures, corresponding to almost double the amount when compared to the former case. An interpretation of these results is that protein interaction with silver ions during silver nanoparticle synthesis and subsequent capping of the AgNPs favors the α -helix and random coil structural elements. The absence of silver ions favors the presence of β -sheets, which can be related to a temperature effect during synthesis and different solvent exposure.

3.2.6. Antibacterial Activity of AgNPs

The antimicrobial activity of the biosynthesized AgNPs was studied against the Gram-negative *E. coli* BL21(DE3) bacterium, monitoring the inhibition of cell growth. Different concentrations of AgNPs suspensions (2.5, 7.5, and 10 mg/L) were added to the cultures (Figure 10a). Bacterial growth curves usually exhibit four phases: the lag phase, the exponential growth phase, the stationary phase, and the death phase. The inhibition effect of the AgNPs was concentration-dependent, with the highest degree of inhibition observed in the culture supplemented with 10 mg/L of AgNPs suspension. The addition of AgNPs, at the concentrations tested in this work, altered the growth kinetics of the cells. Increasing concentrations of AgNPs added to the cultures further delayed the exponential growth phase (the lag phase increased from $\sim 1.3\text{ h}$ for the control to $\sim 4.6\text{ h}$ for the AgNP treated culture), as well as the growth rate. Table 1 summarizes the parameters obtained by fitting the four-parameter Gompertz growth-curve model to the experimental data [27]. The data reveal that the AgNPs synthesized using *M. oleifera* seed cake extracts have a significant antibacterial activity, in agreement with other studies reported in the literature [24,27].

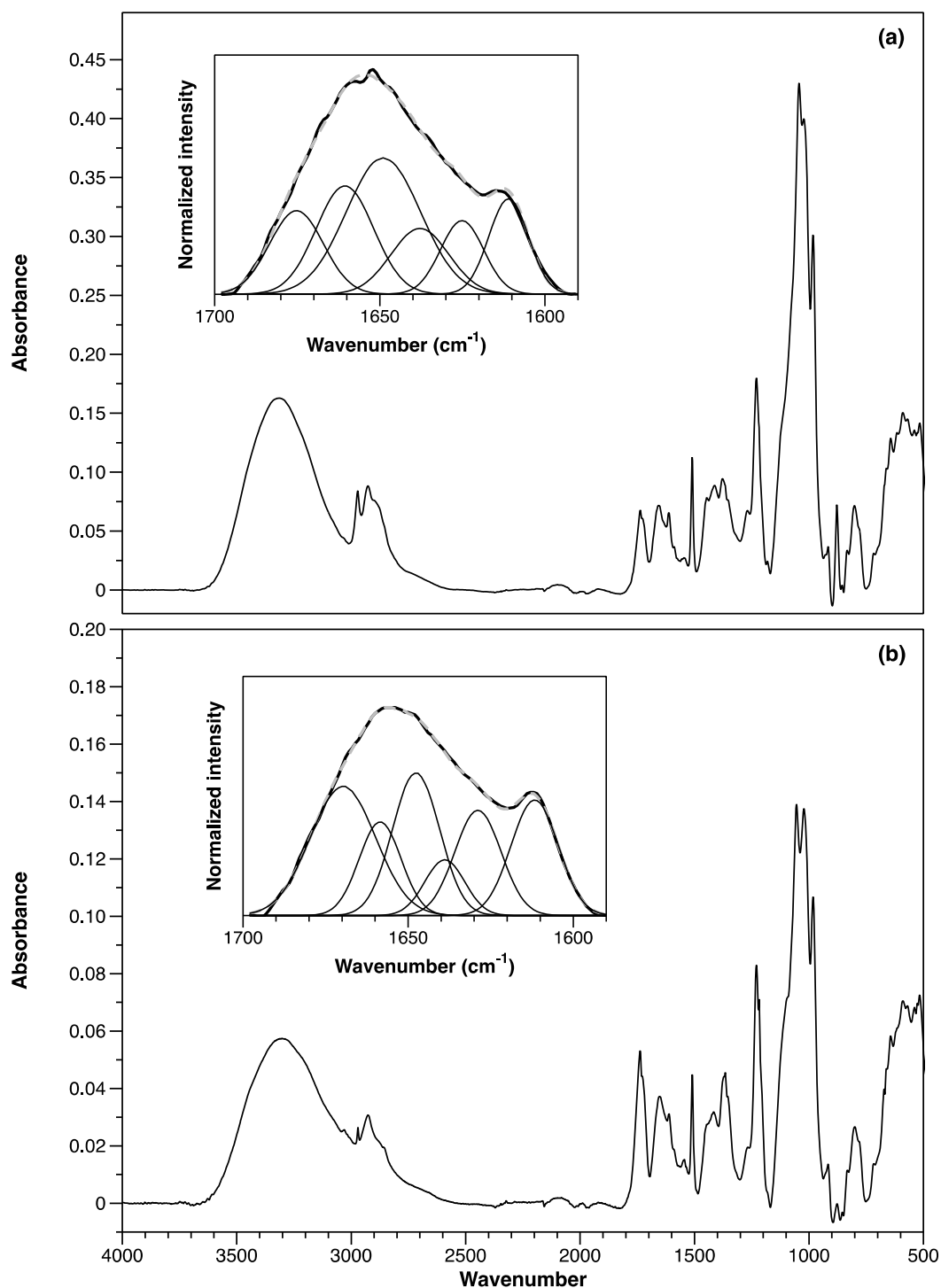


Figure 9. ATR-FTIR spectra of AgNPs (a) and synthesis control (b) reaction products. The insets show the corresponding amide I region of each spectrum presenting the FTIR spectrum (thick black lines) and curve fit (dashed grey lines) using a model containing six Gaussians (thin lines; see Table S3 for parameters).

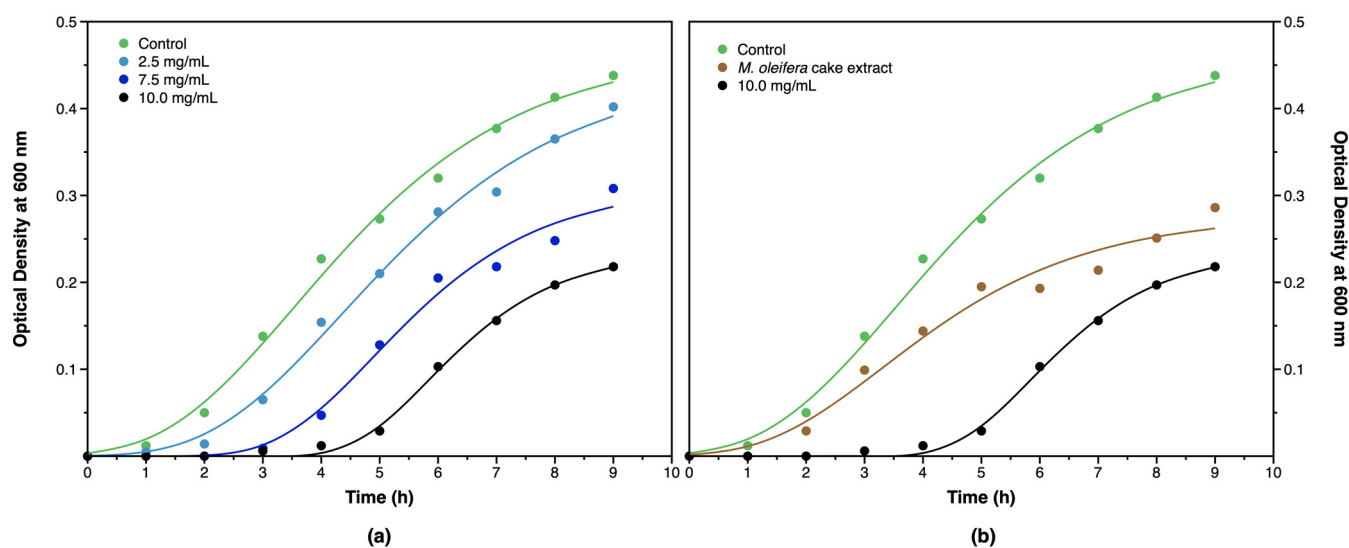


Figure 10. (a) Growth kinetics of *E. coli* BL21(DE3) cultures supplemented with *M. oleifera* AgNPs at different concentrations (2.5, 7.5, and 10.0 mg/L). (b) Comparison of the growth inhibition by 10.0 mg/L AgNPs (dark blue) with the effect of adding an equivalent volume of *M. oleifera* seed cake extract (brown) and the untreated control (green). The theoretical solid lines overlaying the experimental points were obtained by data fitting using the four-parameter Gompertz growth-curve model (see Section 2.5). Experimental data are presented as mean of duplicate measurements. Optical density values of 1:10 dilution of each aliquot.

Table 1. Parameters obtained by fitting the growth curve using the four-parameter Gompertz model.

Culture	λ (h)	μ_{max} (h ⁻¹)	A
Control	1.34 ± 0.17	0.078 ± 0.005	0.469
2.5 mg/mL AgNP	2.11 ± 0.18	0.073 ± 0.005	0.440
7.5 mg/mL AgNP	3.26 ± 0.27	0.070 ± 0.010	0.312
10.0 mg/mL AgNP	4.55 ± 0.10	0.068 ± 0.004	0.237

λ —lag phase duration (h); μ_{max} —maximum specific growth rate (h⁻¹); A is the upper asymptote (theoretical maximum for the maximum optical density).

The inhibitory mechanism by AgNPs is still not fully understood and may involve several modes of actions [24,34,35]: (1) AgNPs attach to bacterial cell walls and membranes through electrostatic interactions, causing structural changes that affect membrane functions, such as transport, enzymatic activity, and ATP production; (2) AgNPs induce the formation of reactive oxygen species that can damage membrane lipids, proteins, and DNA, disrupting several important metabolic processes; and/or (3) impairing the formation of biofilms due to the adhesion of the nanoparticles at the surface of bacterial cells.

To further certify that the growth inhibition observed in the *E. coli* cultures was caused by direct action of AgNPs, the bionanoparticle suspension was replaced by the same volume of *M. oleifera* cake extract and compared with the untreated control (Figure 10b). It is possible to conclude that, although the *M. oleifera* cake extract inhibited cell growth, the nanoparticles had a stronger inhibitory effect, lengthening the lag phase and inhibiting cell growth to a further extent. Remarkably, the extract did not significantly affect the lag phase, suggesting a different inhibition mechanism.

The antibacterial properties of the extract (although less efficient than the ones exerted by the AgNPs) may be due, mainly, to the presence of the protein Mo-CBP₃ (or members of the same family of proteins), known for its antimicrobial properties [12,16,17].

The presented data support the successful synthesis of AgNPs with an average core size of around 127 nm and an effective hydrodynamic diameter of 174 nm due to biocapping. FTIR data point to the nature of a protein biogenic capping agent, and SDS-PAGE

strongly supports that the major protein in the extract is the plant albumin Mo-CBP₃, which possesses eight cysteine residues per molecule. Taken together, we can propose the following mechanism: in a first step, silver ions are reduced and chelated by thiol groups of proteins. In a synergetic effect, other small molecules can assist in the reduction process, such as ascorbic acid, which is highly soluble and can be obtained in seed cake extracts up to millimolar concentration. Ascorbic acid can be oxidized to dehydroascorbic acid, providing a total of two reducing equivalents in two consecutive electron transfers steps. The bound protein can control the nucleation step by avoiding aggregation, stabilizing, and constraining particle growth due to the presence of amine-containing amino acid sidechains. Overall, protein capping will control particle growth and avoid interparticle interactions, resulting in a stable AgNPs preparation.

It also considers the antibacterial mode of action of the AgNPs obtained. Silver nanoparticles are capable of adherence to cell walls and membranes, eventually entering the cell. Once inside, the deleterious mechanisms are vast, ranging from generating reactive oxygen species and free radicals to interacting with sulfur and phosphorus containing compounds or destabilizing ribosome complexes and inhibiting protein synthesis. The protein coating on AgNPs can potentiate all these effects, since Mo-CBP₃ will contribute to membrane permeabilization in what can be considered a synergistic effect.

4. Conclusions

A simple, inexpensive, and environmentally friendly method for the synthesis of AgNPs was successfully carried out using *Moringa oleifera* seed cake aqueous-ethanolic extracts that contain a major protein, most likely Mo-CBP₃. The process is fast, can be easily scaled up and makes use of a *Moringa* residue normally wasted. The synthesis of AgNPs was performed at pH $\simeq$ 5 and 80 °C, producing nanoparticles with an average diameter of 127 ± 24 nm, as determined by SEM. The formation and stabilization of AgNPs were verified by color change of the suspension (from light yellow to bronze brown) and UV-Visible spectroscopy. The zeta-potential and crystalline structure of the synthesized bionanoparticles were also determined.

One of the most important properties of AgNPs is their antimicrobial activity. To evaluate the effect of these AgNPs on the growth kinetics, *E. coli* BL21(DE3) cultures were supplemented with different concentrations of the biosynthesized nanoparticles. The data presented in this manuscript show that the AgNPs significantly inhibited bacterial growth, elongating the lag phase, in a dose-dependent manner.

The antimicrobial activity of AgNPs and of the *M. oleifera* cake extract was also compared. In agreement with the literature, the seed cake extract itself inhibited bacterial growth, although to a much lesser extent. Since the cake extract did not affect the lag phase duration, it is reasonable to infer that its inhibitory mechanism differs from the AgNPs one.

Although the antimicrobial activity of *M. oleifera* extracts is well documented, very few studies are known using seed cake extracts. Adding value to a by-product will contribute to a circular economy by profiting from a product previously discarded as waste, in an environmentally friendly manner. In addition, finding alternative agents to conventional antibiotics is of uttermost importance to fight against resistance of pathogenic bacteria.

It should be noted that much of the focus of the work presented is (i) to ascertain the possibility of using a *Moringa oleifera* subproduct of the oil extraction process (seed cake) to produce a higher added-value product, namely silver nanoparticles; (ii) to produce a simple process that can be used in the absence of any specialized facilities/laboratories and, eventually, in the same location of seed cake production; and (iii) to test possible applications of synthesized AgNPs. Nevertheless, this study raises more questions on the silver nanoparticle synthesis mechanism and antibacterial mode of actions. Further studies are needed to understand the bio-reduction and coating role (and nature) in biosynthesis, as well as the antimicrobial mechanism of AgNPs synthesized from *M. oleifera* seed cake extracts. For example, it will be important to use purified components of the seed cake extract, such as the Mo-CBP₃ protein itself to probe the nature of the coating.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/coatings13081439/s1>, Table S1. Seed cake composition; Table S2. Particle size estimation using DLS spectroscopy; Table S3. Secondary structure estimation using FTIR data.

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