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Green Synthesis of Silver Nanoparticles from *Phaseolus vulgaris* L. Extracts and Investigation of their Antifungal Activities

Phaseolus vulgaris L. Özütlerinden Gümüş Nanoparçacıkların Yeşil Sentezi ve Antifungal Etkinliklerinin İncelenmesi

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ABSTRACT

The aim of this study was to synthesize silver nanoparticles (AgNPs) using leaf, root, and stem extracts of *Phaseolus vulgaris* L. (Yunus-90) and elucidate their antifungal activities. In this regard, the prepared AgNPs have been characterized by using UV-vis, FT-IR, TEM, SEM, and DLS techniques. Then, the antifungal activity of both synthesized and commercially purchased AgNPs was investigated via (i) agar well diffusion, (ii) fungal colony morphotype diversity, (iii) inhibition of hyphae and (iv) minimum inhibition concentration (MIC) analyses against *Colletotrichum sp.*, *Fusarium oxysporum*, *Fusarium acuminatum*, *Fusarium tricinctum*, *Fusarium graminearum*, *Fusarium incarnatum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Alternaria alternata*. The AgNPs derived from the leaf extract displayed significantly higher levels of antifungal activity relative to the AgNPs prepared from the root and stem extracts. The commercial AgNPs also displayed lower antifungal activity than their green equivalents synthesized in this research, and even a low (~50 µg/mL) concentration of synthesized AgNPs was found to be effective in suppressing the growth of *Fusarium tricinctum* and *Colletotrichum sp.*

Key Words

Phaseolus vulgaris L., green synthesis, silver nanoparticle (AgNP), antifungal activity

ÖZ

Bu çalışmanın amacı, *Phaseolus vulgaris* L.'nin (Yunus-90) yaprak, kök ve gövde özütlerini kullanarak gümüş nanopartiküllerini (AgNPs) sentezlemek ve antifungal aktivitelerini ölçmektir. Bu bağlamda, hazırlanan AgNP'ler UV-vis, FT-IR, TEM, SEM ve DLS teknikleri kullanılarak karakterize edilmiştir. Ardından hem sentezlenen hem de ticari olarak satın alınan AgNP'lerin *Colletotrichum sp.*, *Fusarium oxysporum*, *Fusarium acuminatum*, *Fusarium tricinctum*, *Fusarium graminearum*, *Fusarium incarnatum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* ve *Alternaria alternata*'ya karşı antifungal aktivitesi; (i) agar kuyu difüzyonu, (ii) mantar kolonisinin morfolojik çeşitliliği, (iii) hif inhibisyonu ve (iv) minimum inhibisyon konsantrasyonu (MIC) analizleri ile araştırılmıştır. Yaprak özütlerinden türetilen AgNP'ler, kök ve gövde özütlerinden hazırlanan AgNP'lere göre önemli ölçüde yüksek antifungal aktivite sergilemiştir. Ticari AgNP'ler ayrıca bu araştırmada sentezlenen yeşil eşdeğerlerinden daha düşük antifungal aktivite göstermiştir. Ayrıca, sentezlenen AgNP'lerin düşük (~ 50 µg/mL) konsantrasyonunun bile *Fusarium tricinctum* ve *Colletotrichum sp*'nin gelişimini baskılamada etkili olduğu bulunmuştur.

Anahtar Kelimeler

Phaseolus vulgaris L., yeşil sentez, gümüş nanoparçacık (AgNP), antifungal aktivite.

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INTRODUCTION

In the last decade, advanced biological techniques based on phyto-nanobiotechnology, including the utilization of herbal extracts, have made significant progress as a sub-branch of nanotechnology [1–3]. The procedures used in this context have mainly a "green" synthesis perspective, thus providing a more efficient and environmentally-friendly approach, saving energy, as well as being more economical when compared to traditional materials [4,5]. Researchers are now able to generate end products with pre-designed and diversified functional properties by interpreting nanoparticles, which are at the core of the field of nanotechnology. Therefore, a significant number of researches has been carried out in the literature about "green" synthesis and characterization of the metal nanoparticles, especially for gold (Au), silver (Ag), platinum (Pt), and palladium (Pd) [6–9].

It should also be mentioned that AgNPs prepared by methods based on chemical or phyto-nanobiotechnology have been found extensive use in the fields of biosensor [10–14], photonic [15,16], photocatalysis [17–20], pharmaceutics [21–26], and microelectronics [27–31]. Besides, AgNPs have been shown to have conspicuous antifungal [32–34], antimicrobial [35–39], antiviral [40–44], antibacterial [45–49], anti-inflammatory [50–53], and anticancer [54–58] effects. In this scope, soil microorganisms and plant extracts have great potential for the synthesis of target AgNPs as important bioagents [59–63]. Almost all nanobiotechnology applications emphasize the need for new approaches to acquire the resulting nanoparticles through an effective and environmentally friendly synthesis process.

Nowadays, organic and inorganic reducing agents, such as sodium citrate [64,65], sodium ascorbate [66], sodium borohydride [67], and elemental hydrogen [68], are widely used in the synthesis of AgNPs. In general, these reducing agents provide the reduction of positively charged silver (I) ions to the metallic form silver (0). In this study, in contrast to the toxic reducing agents used in chemical methods, extracts from bean plants in the aqueous medium were used. Following this strategy, we have sought to develop an environmentally friendly and inexpensive synthetic approach to obtain target AgNPs. The efficacy of obtaining AgNPs from different parts of *Phaseolus vulgaris* L. (Yunus-90) was therefore investigated and compared with the activity of synthesized nanoparticles against different fungal plant pathogens. The methodology used in these experiments is thought to give a unique value to research as it offers a different method to current examples in literature.

MATERIALS and METHODS

Plant Growth, Sampling and Extraction Procedures

The seeds (common bean 'Yunus-90 cv.') supplied by the 'Transitional Zone Agricultural Research Institute, Eskişehir, Turkey,' were surface sterilized in hypochlorite solution (5%, v/v) for 5 min. The sterilized seeds were then hydroponically grown in pots containing modified 1/10 Hoagland solution (0.2 L) until the plants reach full maturity, also known as the trifoliate leaf stage. Following this step, the root, stem, and leaf tissues considered for AgNPs synthesis were harvested [69]. Each tissue sample (~200 g) was washed with the ddH2O three times and boiled in ddH_2O (200 mL). The extracts were incubated at 60°C for one hour in a water bath. Upon incubation, plant extracts were filtered using Whatman filter paper (No: 1), and the final extracts were held at -20°C during the investigation (Fig S1).

Preparation of the Silver Nanoparticles

Plant extract (10 mL) was allowed to warm up to room temperature (RT) to prepare AgNPs. The extract was then combined with AgNO3 (1 mM, 100 mL) and agitated for an additional 45 min at RT. The resultant mixture was centrifuged at 10,000 rpm for 30 min to precipitate the intended AgNPs (Fig. S2). The collected nanoparticles were transferred to the microfuge tubes (1.5 mL) and washed three times with ddH2O. After drying in the oven at 65°C, the concentration of AgNP obtained was determined (approximately 1.8 to 2.5 mg per milliliter of leaf extract).

Structural Characterization of the Silver Nanoparticles

The target AgNPs obtained from the leaf, stem, and root extracts were firstly characterized by using Ultraviolet-visible spectroscopy (UV-vis), and Fourier Transform Infrared Spectroscopy (FT-IR) techniques. The AgNPs obtained from the leaf extracts were also investigated by Transmission Electron Microscopy (SEM), Scanning Electron Microscopy (SEM) and Dynamic Light Scattering Spectroscopy (DLS) techniques. In this scope, UV-vis spectra were taken using a Cary 100 Scan UV-vis spectrophotometer (Varian, Germany) in quartz cuvet-

tes (3,500 µL, 10 mm light path, Hellma 110-QS) at a scanning rate of 1 nm at a range of 300-800 nm. While UV-vis spectra were taken, ddH2O was used as a solvent to show consistency with the experimental conditions. Perkin Elmer Spectrum 100 FT-IR spectrometer (Perkin-Elmer Inc., Norwalk, CT, USA) was used to obtain the corresponding FT-IR spectra by applying the ATR kit. The surface morphology of AgNPs was performed by using an FEI, Tecnai G2 F30 (LaB6 type electron gun, 20x-910.000x magnification axis, 0.2±0.04 nm point resolution power) (Thermo Fisher Scientific, Hillsboro, OR, USA) microscope (TEM) and a Zeiss EVO-40 Model (500V-30kV High Voltage; SE, BSD, EDX, VPSE detector, W-filament electron gun) (Carl Zeiss, Oberkochen, Germany) electron microscope (SEM), respectively. Malvern ZetaSizer NanoZS90 (Malvern instruments Ltd., UK) was also used for the size analysis of the AgNPs at 25°C for a measurement period of 20 seconds.

Antifungal Activity Studies

Fungal cultures

Colletotrichum sp., Fusarium oxysporum, Fusarium acuminatum, Fusarium tricinctum, Fusarium graminearum, Fusarium incarnatum, Rhizoctonia solani, Sclerotinia sclerotiorum, and Alternaria alternata fungal pathogen isolates which were known to cause pathogenic effects in common bean were obtained from Ankara University, Biotechnology Institute. Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) was used to prepare corresponding active cultures. Fungal colonies were inoculated on PDA plates, and Petri dishes were incubated at 25°C for 24 to 48 h, and the growth of isolates was monitored at regular intervals. Active fungal cultures were maintained for use in subsequent studies.

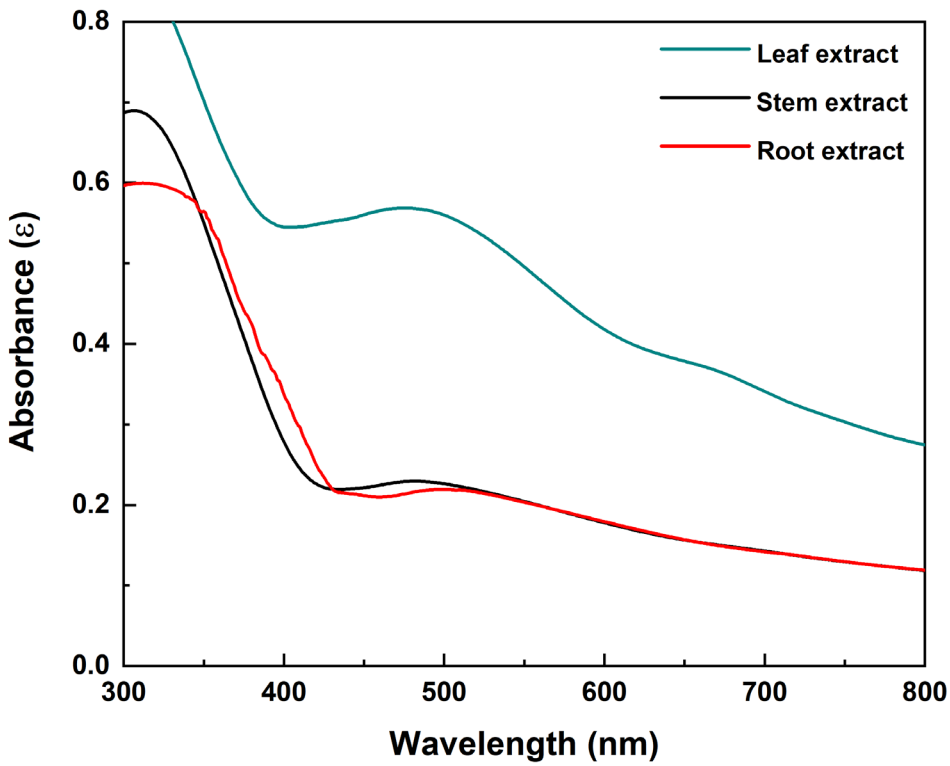

Figure 1. The UV-vis spectra of the AgNPs synthesized by using *Phaseolus vulgaris* L. leaf, stem, and root extracts.

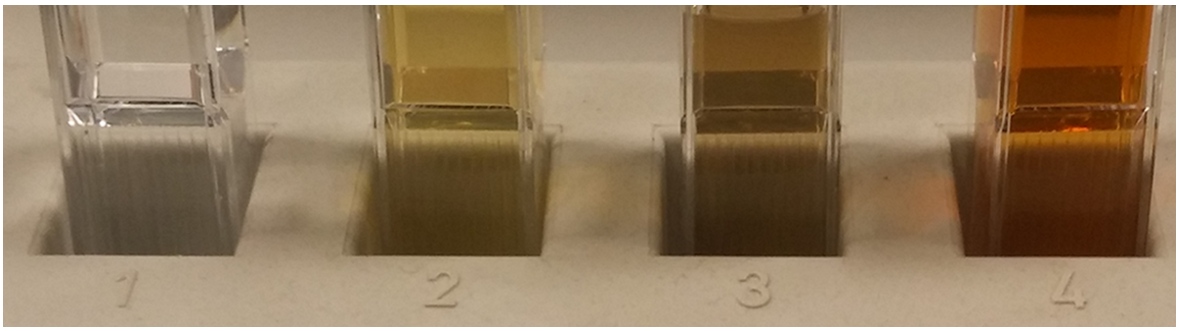

Figure 2. Time-dependent formation of the AgNPs in *Phaseolus vulgaris* L. leaf extracts.

Determination of antimicrobial activity by agar well diffusion method

According to a previous study, the antimicrobial effect of AgNPs on fungal pathogens was measured by the implementation of a typical agar well diffusion technique [70]. Consequently, the antimicrobial activity of the synthesized AgNPs utilizing different parts of the common bean (root, stem and leaf) has been thoroughly investigated. To this purpose, active fungal colonies were harvested by sterile loops, and the samples were transferred to the tubes containing 5 mL of sterile saline (0.85% NaCl). The suspensions were prepared based on the 0.5 MacFarland standard. Subsequently, 100 uL of these samples were taken and inoculated into the tubes comprising 5 mL of soft PD (0.75% agar) and vortexed. After this phase, soft media spread to the surfaces of the PDA-containing Petri dishes. The Petri dishes were allowed to dry for 10 min before treatment. 9 mm wells were drilled on the agar surfaces under aseptic conditions. 100 uL of AgNPs prepared from root, stem, and leaves and adjusted to 1 mg/mL concentration were transferred into the wells. Petri dishes were finally incubated at 25°C for 24 h. After the incubation, inhibition zone diameters were measured in "mm" and the resulting antimicrobial activity was then determined.

Determination of inhibition in the development of the fungal colony

AgNPs prepared from the plant leaves were added to the prepared PD agar (20 mL) with an increasing concentration of 0.125, 0.25, 0.5, 1, 2 and 4 mg/mL before dispensing to the Petri dishes. The media containing the required concentration of AgNPs were then poured into the Petri dishes and allowed to dry. In this study, the susceptible fungal isolates inhibited by synthesized AgNPs *(Fusarium oxysporum, Fusarium graminearum, Fusarium incarnatum, Fusarium acuminatum, Fusarium tricinctum, Colletotrichum sp.)* were used. The culture of inoculation has been developed, as already mentioned. Active crops have been found at the core of the PDA dishes containing different concentrations of AgNPs. Particle-free PDA media have been used as a positive control group and all Petri dishes were incubated at 25°C for 24 to 48 h. The diameters of the colonies were measured in mm. The percentages of declines in the growth of the colony were calculated based on the growth of the colony in the positive control groups [71].

Microscopic analysis of regression in hyphae development

The preparation of fungal samples from test groups with the highest particle concentration and control groups was carried out by microscopic sliding. The samples were stained with methylene blue and examined at 40X and 100X magnifications under a light field microscope (Leica, Germany).

Minimum inhibition concentration (MIC) tests

In this research, the susceptible fungal isolates to AgNPs are favored throughout accordance with previous antifungal activity studies. The cultivation of fungal isolates was carried out in YPG medium (Yeast-Peptone-Glucose; Yeast extract 10 g/L, Peptone 20 g/L, Glucose 20 g/L). The appropriate incubation times were considered for each isolate at 25°C for the growth of cultures (in the case of *Fusarium graminearum, Fusarium incarnatum, Fusarium acuminatum, Fusarium oxysporum;* 48 h, for *Fusarium tricintum and Colletotrichum* sp; 96 h). The activated cultures were then used for MIC test. In this assay, the antifungal activity of AgNPs obtained through green synthesis from plant leaves and commercially available AgNPs (Merck, Product Code: 730785, Germany). The culture suspensions containing both green and chemically synthesized AgNPs adjusted to final (0.0, 0.312, 0.625, 1.25, 2.5, and 5.0 mg/mL) concentrations by sequential dilution using YPG medium (100 µL) in

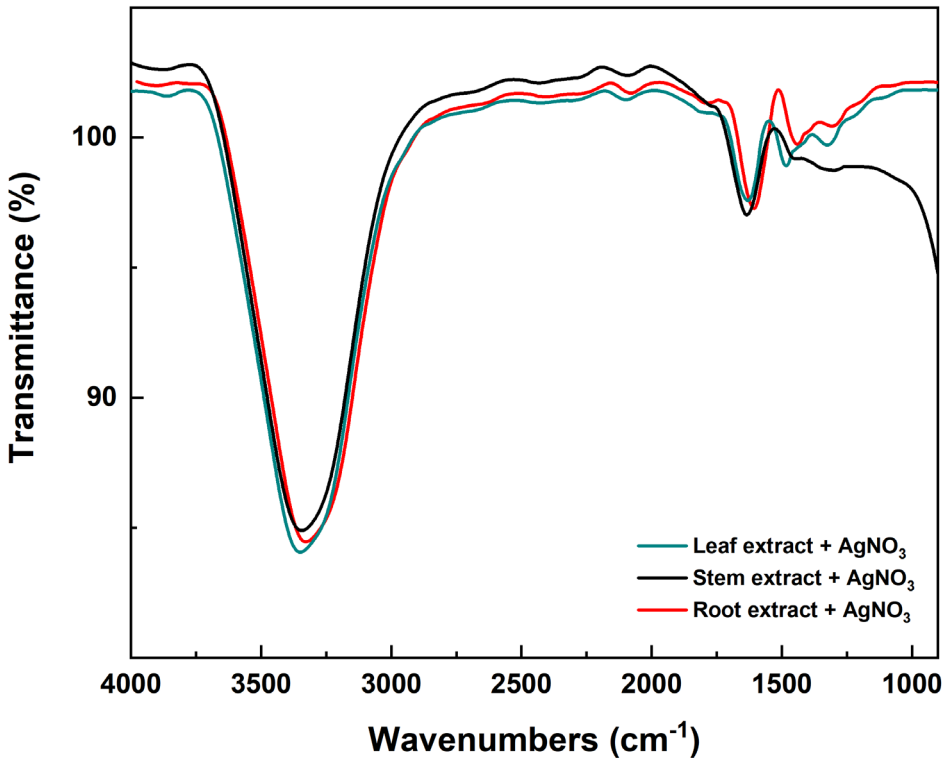

Figure 3. FT-IR spectra of leaf, stem, and root extracts containing AgNPs .

U-bottom 96-well microtiter plate wells. Only inoculum-containing wells were used as positive controls without AgNPs, while only medium-containing wells were designed as negative wells. The activated fungal cultures were vortexed at the highest intensity before inoculation, and 10 µL of the culture suspensions were inoculated into the test and positive control wells. Plates were incubated at 25°C for 48 and 96 h. At the end of the incubation periods, the culture suspensions in the wells were suspended by pipetting and 50 µL these suspensions were transferred and spotted onto PDA plates by drop plate method. Petri dishes were allowed to incubate at 25°C for 48 h, and the concentrations that completely inhibited the fungal growth were determined to be MIC values at the end of the incubation [72].

RESULTS and DISCUSSION

The synthesis of AgNPs from plant extracts was carried out using a phyto-nanotechnological process. The average amount of nanoparticles per milliliter of leaf extract ranged from 1.8 mg to 2.5 mg. On the other hand, the extracted nanoparticle mass per milliliter of stem and root extract was estimated to be 0.8 mg and 0.3 mg, respectively. UV-vis spectroscopy was used for the structural analysis of the nanoparticles, and according to the results, in the natural bio-reducing extract medium of *Phaseolus vulgaris* L., silver (I) cations were reduced to neutral silver (0) and, eventually AgNPs were produced. As shown in Figure 1, the broadband observed in the 450-480 nm range, especially in leaf extract, is characteristic for polydispersed AgNPs in solution. Similar band formation can be identified for stem extract even if it is weak; however, no substantial band formation is observed in root extract in this region (Figure 1).

According to previous literature research, AgNPs, whose concentration of the solution increases over time, can be qualitatively observed by taking a yellow-brown color in their solutions due to vibration motions according to the theory known as surface plasmon resonance [73–75]. The surface plasmon resonance is a physical

phenomenon that arises as a result of the vibration motions of the plane-polarized light on the metal surface during its reflection. In general, color changes were observed with the application of silver nitrate solution to the colorless plant extracts, which initially approached yellow and then became brown in this analysis (Figure 2, Fig S3).

Plant extracts constitute a rich environment of amino acids and proteins. The carbonyl groups of these complex molecules surround AgNPs and enhance particle stability. Once their environmental stability has been enhanced, the possible agglomeration is avoided, and the growth of particle size is also inhibited. According to this observation, leaf, root, and stem extracts have, according to the FT-IR study, also been shown to consist of similar functional groups (Figure 3).

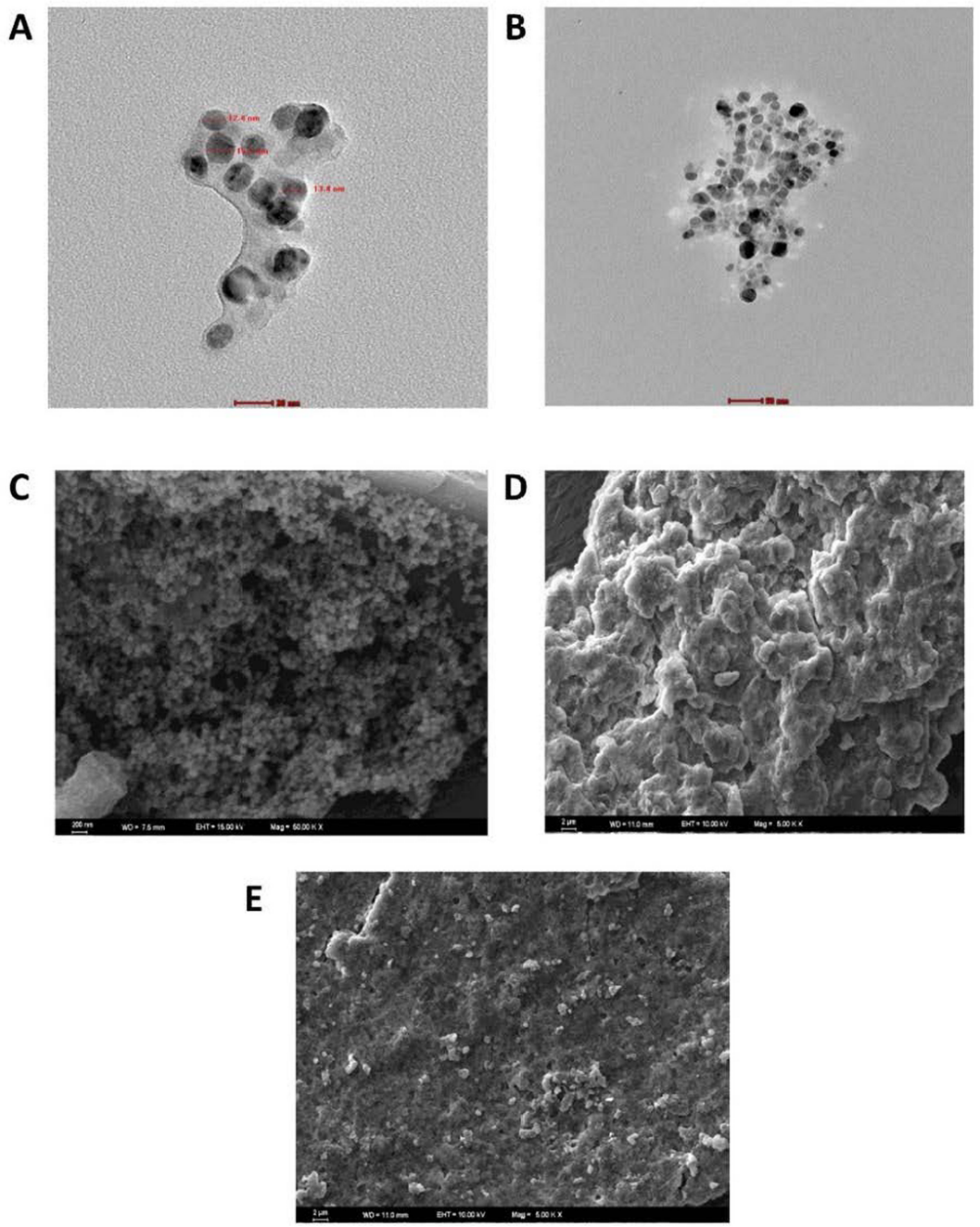

Figure 4. TEM (A, B) and SEM (C, D, E) images of the AgNPs in leaf extracts.

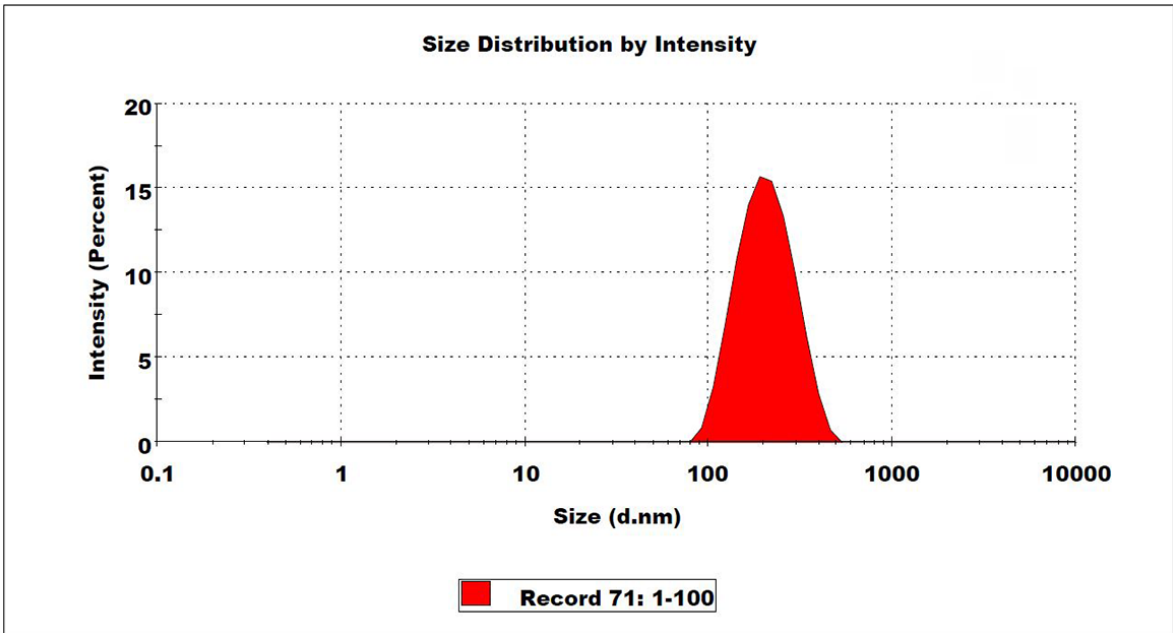

Figure 5. DLS analysis of the AgNPs obtained from leaf extract.

In the FT-IR spectrum, the strong and broadband in the range of 3500 to 3200 cm^{-1} belongs to the O-H stretch, which is typically observed in phenols and hydrogen-bonded alcohols. The weak peak observed at 2086 cm^{-1} corresponds to the aromatic C-O stretch of the polyol groups. The relatively sharp peak at 1636 cm^{-1} shows off-plane bending vibrations in substituted ethylene systems. As mentioned before, the carbonyl (C=O) functional group and the hydroxyl (OH) groups formed in the structure of the plant extract and the reduction of this group have the potential to bond firmly by interacting with metal nanoparticles. In other words, AgNPs are surrounded by these biostructures like a capsule, thus reducing the potential of metal particles to come together to form agglomeration, increasing the stability of the solution medium [53].

TEM images from the leaf (Figure 4), stem and root (Fig S4) extracts particles show that the synthesized AgNPs have a very smooth surface morphology and spherical geometry. The nanoparticles were found to have an average diameter of 12 to 16 nm with highly uniformly dispersed, as shown in the TEM images. This leads to the conclusion that some essential bioorganic molecules in the plant extract behave as a ligand that stabilizes the AgNPs in the medium. In other words, the components in the plant control the growth and aggregation kinetics of the nanoparticles.

Finally, the DLS technique was used for the structural characterization of nanoparticles, and particle size distributions in extracts were examined. Measurements, where the average particle size is estimated to be 188 nm, confirming that AgNPs are present in small clusters and do not contain agglomerates (Figure 5).

Antifungal Activity

The antifungal effects of AgNPs from different parts of the common bean were measured using the technique of well diffusion agar as shown in Table 1.

It was concluded that the AgNPs obtained from leaf extracts demonstrated higher levels of antifungal activity than nanoparticles derived from the root and stem extracts. After applying nanoparticles to leaf extracts, most of the wells observed the development of an inhibition zone. Nonetheless, after treatment with root and stem extracts, almost no inhibition zone formation was observed (Table 1). The formed inhibition zone images were also provided in Table 2.

Table 1. Evaluation of the antifungal activity of silver nanoparticles.

Species Name	Leaf	Stem	Root
Fusarium acuminatum	14 mm	11 mm	-
Fusarium incarnatum	18 mm	-	-
Fusarium graminearum	12 mm	-	-
Fusarium tricinctum	15 mm	-	-
Fusarium oxysporum	13 mm	-	-
Rhizoctonia solani	-	-	-
Sclerotinia sclerotiorum	-	-	-
Colletotrichum sp.	18 mm	-	-
Alternaria alternata	-	-	-

Through doing this, the zone diameters of the corresponding fungal species were determined to assess the inhibition of the growth of the fungal colony. As a result, the growth of fungal pathogens was reduced at higher concentrations of AgNPs. As shown in Table 3, the development of *Fusarium tricinctum* and *Colletotrichum sp.* species was inhibited. On the other hand, when compared with the control group, the most of the fungal species were shown to have decreased zone diameters with increased concentrations of AgNPs (Table 3).

The zone diameters of fungal pathogens exposed to different concentrations of nanoparticles have been measured, and their developmental tendencies have been determined. Table 4 indicates the concentration-dependent shifts (%) in the diameter of the zone.

Table 2. Inhibition zone formation by agar well diffusion assay (Y: leaf, G: stem, K: root extracts)

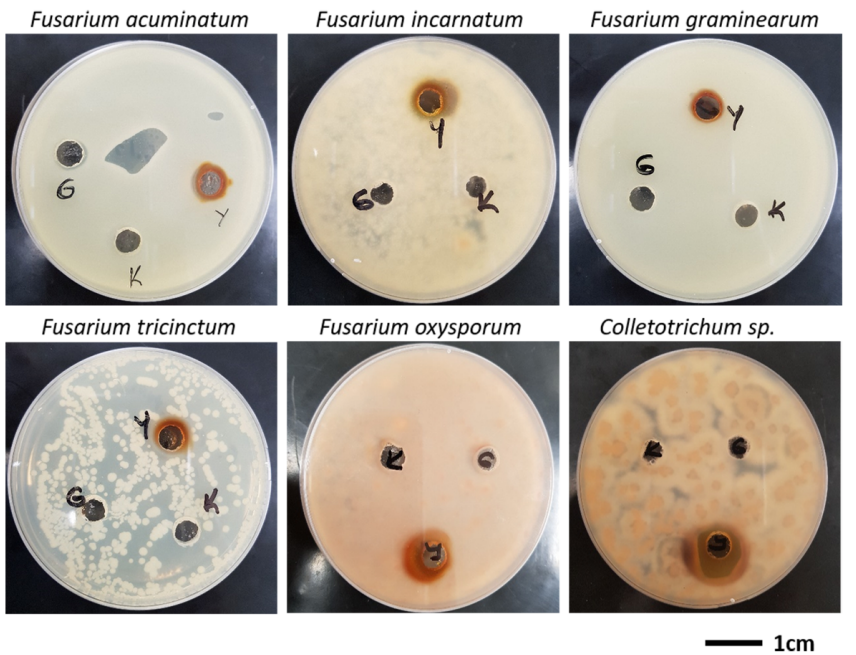

Table 3.Comparison of the development of fungal colonies in medium containing a high concentration of AgNPs (right) and the control group (left).

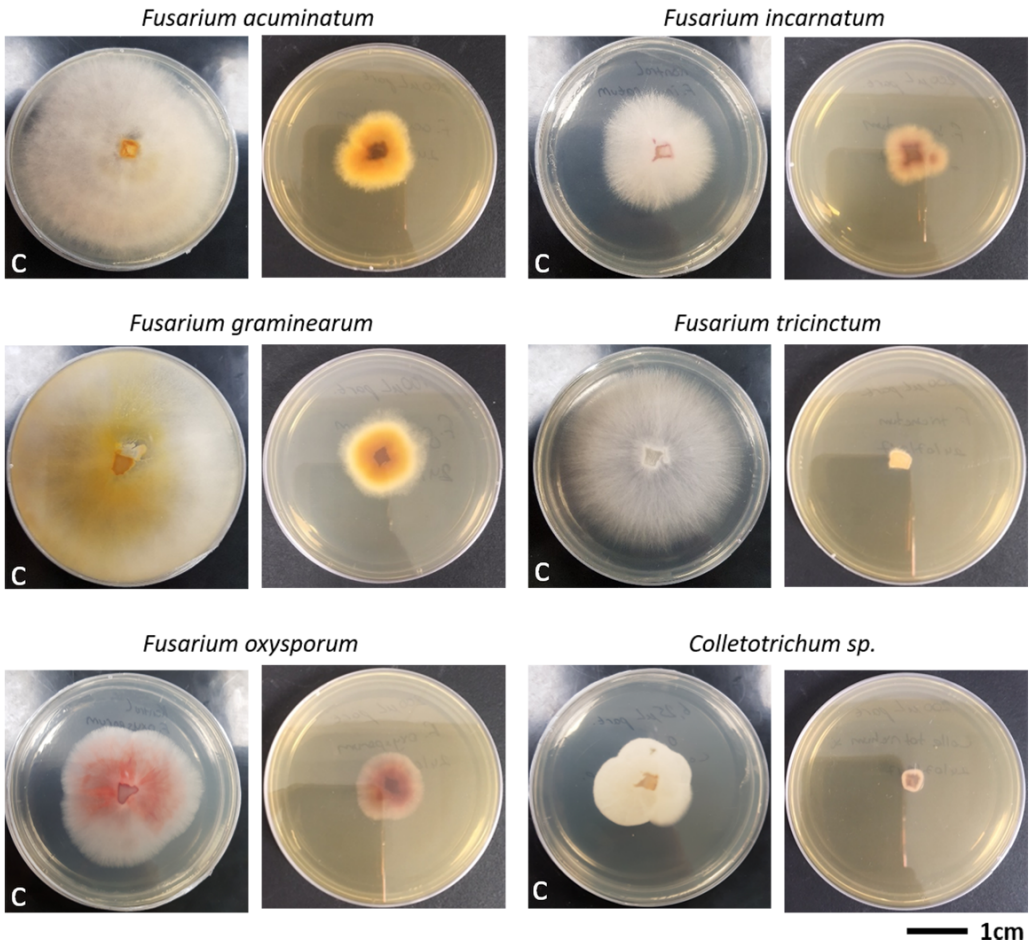

Table 4. The regression in colony diameters of fungal species with an increased concentration of AgNPs.

AgNP concentration	6.25 µg/mL	12.50 µg/mL	25.00 µg/mL	50.00 µg/mL	100.00 µg/mL	200.00 µg/mL
Fungal species	Colony regression (mm; %)					
Fusarium acumitnatum	3 mm; 3.90%	3mm; 3.90%	6 mm; 7.79%	23 mm; 29.87%	46 mm; 59.74%	49 mm; 63.63%
Fusarium incarnatum	4 mm; 10.53%	4 mm; 10.53%	4 mm; 10.53%	5 mm; 13.16%	18 mm; 47.37%	18mm; 47.37%
Fusarium graminearum	0 mm; 0%	0 mm; 0%	0 mm; 0%	0 mm; 0%	48 mm; 60.76%	58 mm; 73.42%
Fusarium tricinctum	20mm; 30.77%	29 mm; 44.61%	46 mm; 70.76%	58 mm; 89.23%	60 mm; 92.30%	65 mm; 100%
Fusarium oxysporum	0 mm; 0%	0 mm; 0%	0 mm; 0%	2 mm; 4%	25 mm; 50%	29 mm; 58%
Colletotrichum sp.	0 mm; 0%	0 mm; 0%	0 mm; 0%	5 mm; 19.23%	15 mm; 57.69%	26 mm; 100%

Table 5. Light microscopy analysis of hyphae in the medium containing a high concentration of AgNPs (right) and the control group (left).

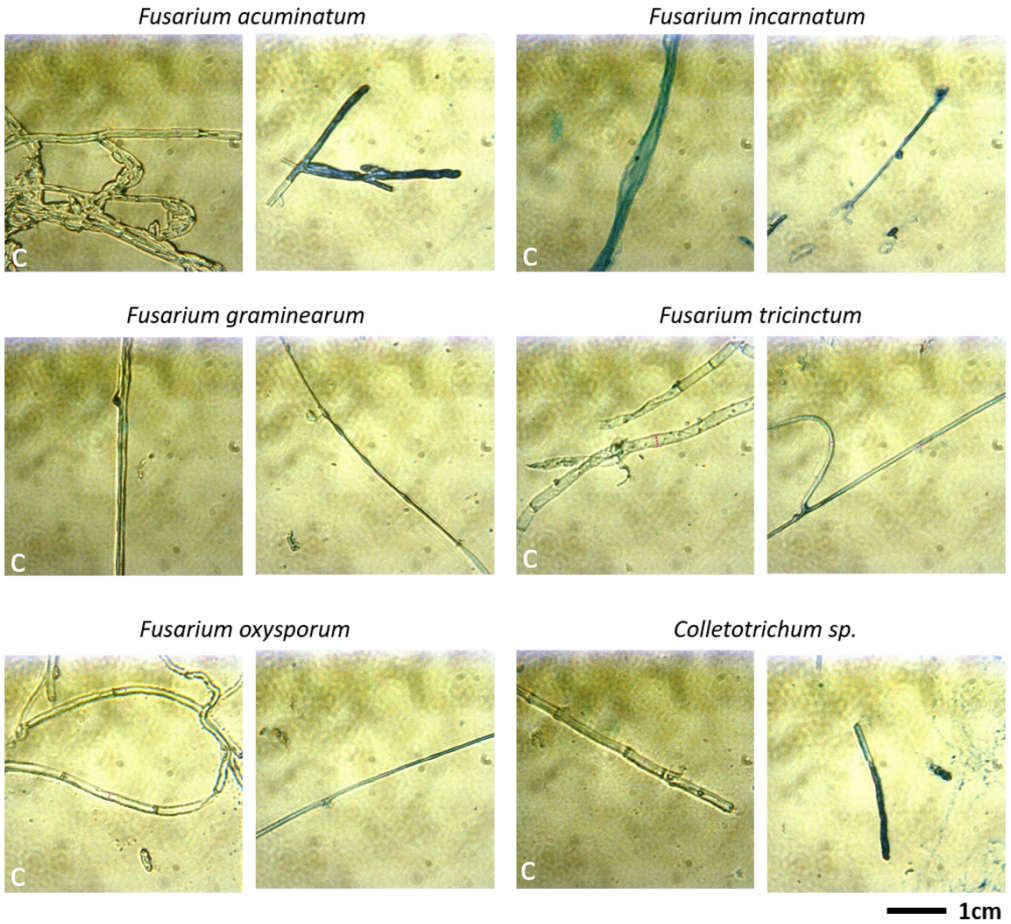

Table 6. Evaluation of the antifungal activity of silver nanoparticles.

Fungal Species	AgNPs (synthesized in this study	AgNPs (commercially purchased)
Fusarium acuminatum	0.312 mg/mL ≥	5.0 mg/mL
Fusarium incarnatum	0.312 mg/mL ≥	1.25 mg/mL
Fusarium graminearum	1.25 mg/mL	1.25 mg/mL
Fusarium tricinctum	0.312 mg/mL ≥	0.312 mg/mL
Fusarium oxysporum	0.312 mg/mL ≥	0.312 mg/mL
Colletotrichum sp.	0.312 mg/mL ≥	0.625 mg/mL

The reduced growth rate observed for the investigated fungal species was associated with the concentration of AgNPs. 100 and 200 µg/mL particle concentrations were shown to slow down the pathogen development in a more effective way than lower concentrations. Surprisingly, this reduction was also valid for lower concentrations (25 and 50 µg/mL) of silver nanoparticles in Fusarium tricintum (Figure 5). Hyphae extracts of fungal pathogen culture collected after 48 h is treated with methylene blue. Subsequently, the silver nanoparticle-containing (200 µg/mL) media and control group (without nanoparticles) samples were compared in terms of antifungal impact. Based on these results, the hyphae diameter of all pathogens is significantly increased with an increased concentration of the particle suggesting inhibition of pathogen dissemination (Table 5).

The objective of the MIC analysis was to determine the minimum concentration of particles required to inhibit the growth of pathogenic fungal species. Therefore, a comparable MIC analysis was carried out between the synthesized AgNPs in the current study with the commercially acquired equivalent (Merck, Product Code: 730785, Germany) to shed light on antifungal activity alterations.

Based on the data obtained, the synthesized AgNPs were found to suppress pathogen development except for *Fusarium graminearum* at lower concentrations than its commercial counterpart (Table 6).

CONCLUSION

In this study, the target AgNPs have been successfully prepared by using a green, environmentally friendly, cost-effective, and virtually acceptable leaf, stem, and root parts of the common bean. The structural characterization of the synthesized AgNPs was carried out by using appropriate spectral techniques. In the following stage, high anti-fungal activity was found even in low concentrations for the synthesized AgNPs, especially in the case of *Fusarium tricinctum*. In other words, synthesized AgNPs were shown to have a considerable potential to be used for suppressing several fungal plant pathogens. Besides that, the nanoparticles obtained from leaf extracts were determined to have a more effective antifungal activity than those from root and stem. Therefore, we suggest that the protection of the economically important plants, such as common beans against the specific fungal pathogens, could be performed following the marketing of these newly synthesized green AgNPs.

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