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Biogenic Synthesis of Silver Nanoparticles Using *Pimpinella anisum L* Seed Aqueous Extract and Its Inhibitory Action against Some Phytopathogens

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Authors' contributions

This work was carried out in collaboration among all authors. All authors designed the study and wrote the protocol. Authors TGMM and AFAER performed the study and wrote the first draft of the manuscript. Author TGMM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Biogenic synthesis of silver nanoparticles (AgNPs) using plant extracts has become a promising substitute for the conventional chemical synthesis method. The present study aims to develop a simple, cost-effective, environmentally friendly method for silver nanoparticles (AgNPs) synthesis using seed aqueous extract of (*Pimpinella anisum L*.) as a reducing agent at room temperature. Characterization of AgNPs was done by UV−visible spectroscopy, high-resolution transmission electron microscopy (HR-TEM), Fourier transform infrared (FTIR) spectroscopy, and selected area electron diffraction (SAED). Phytochemical analysis was performed to determine the phytochemicals responsible for the reduction and capping of the biosynthesized AgNPs. The antibacterial activity of AgNPs was checked against some important phytopathogenic bacteria (*Agrobacterium tumefaciens, Erwinia amylovora, Pectobacterium carotovorm* subsp. *carotovorum*, *Pseudomonas lachrymans, Ralstonia solanacearum*) and *Pseudomonas tolaasii,* Gram-negative

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bacteria causing bacterial blotch on cultivated mushrooms (*Agaricus bisporus*). The UV-Vis spectrum of the synthesized silver colloidal solution showed a strong absorption band at 441 nm, HR-TEM analysis revealed that AgNPs were spherical with an average size of 15 - 37 nm, Moreover, the synthesized silver nanoparticles have shown strong antibacterial activity against all tested pathogenic bacteria, by measuring the inhibition zone using the agar well diffusion method. The obtained results revealed that biosynthesis of AgNPs using plant extract is a promising method in agricultural applications.

Keywords: Antibacterial activity; silver nanoparticles; greensynthesis; Pimpinella anisum seeds; phytopathogenic bacteria.

1. INTRODUCTION

Nanomaterials describe the materials of which nanoscale materials between 1 and 100 nanometers [1] and [2]. There are many methods for producing nanomaterials, including chemical and physical methods such as chemical reduction [3] and thermal evaporation [4]. Most of these methods are very expensive and involve the use of toxic and dangerous chemicals that may pose potential environmental hazards [5] and [6]. Green synthesis of nanoparticles using plant extracts provides advantages chemically and physically way because it is a fast, one-step, low-cost, environmentally friendly method; furthermore, there is no need to use high pressure, energy, temperature, and toxic chemicals [7-10].

Pimpinella anisum L. (Apiaceae) is one of the most common and important medicinal plants cultivated in Egypt. Anise fruits have been used in medicine as carminatives, sedatives, tranquilizers, flavors, aromatic sweets, and chewing gum, and as a mild exfoliant [11-16]. Hence, anise seeds are usually recommended as antioxidants, antiseptic, antispasmodic, antibacterial, fungicidal, in addition to their cosmetic applications [14,17-21]. Anise seeds contain 1.5-6.0% of the volatile mass of volatile oil mainly consisting of trans-anethole and across the mass of 8-11% of fats rich in fatty acids, such as palmitic and oleic acid, as well as about 4% of carbohydrates and 18% of protein [22]. To date, there are no reports of antibacterial activity against plant pathogenic bacteria of silver nanoparticles that are made using (*Pimpinella anisum L*.) seed aqueous extract.

This work was aimed at emphasizing the green approach to synthesize AgNPs using *P. anisum* seed extract. The green synthesized AgNPs have been characterized by UV-Visible spectroscopy, FTIR, HR-TEM, and SAED as well as the study of antimicrobial activity against certain plant pathogenic bacteria.

2. MATERIALS AND METHODS

2.1 Materials

Fresh, healthy *P. anisum* seeds were purchased from Harraz store (Agricultural Seeds, Spice and Medicinal Plants Company, Cairo, Egypt). Silver nitrate was purchased from Windsor Laboratories Limited, UK. Nutrient agar was purchased from Loba Chemie (PVT, LDT, India). Sodium phosphate dibasic dodecahydrate and Sodium phosphate monobasic dihydrate were purchased from Oxford laboratory Reagents, India. De-ionized water obtained by a Milli-Q water purification system was used for all experiments. Five isolates of plant pathogenic bacteria (*Agrobacterium tumefaciens*, *Erwinia amylovora*, *Pectobacterium carotovorm* subsp. *Carotovora* formerly referred to as *Erwinia carotovora subsp*. *carotovora*, *Pseudomonas lachrymans,* and *Ralstonia solanacearum*) in addition to *Pseudomonas tolaasii* (Gram-negative bacteria causing bacterial blotch on cultivated mushrooms, *Agaricus bisporus*) were obtained from the Department of Bacterial Diseases Research, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

2.2 Preparation of Seed Extract

Fresh and healthy *P. anisum* seeds were washed in running water and finally rinsed with de-ionized water until no foreign materials remained. To prepare the *P. anisum* aqueous seed extract, 10 g of seeds were added to 100 ml of de-ionized water at 60°C for 5 min and then allowed to cool at room temperature. The solution was then filtered using Whatman No. 1 filter paper and stored at 4°C for further use, being used for one week due to the gradual loss of plant extract viability for prolonged storage [23,24].

2.3 Phytochemical Analysis of Seed Extract

The initial qualitative phytochemical examination of the various phytochemical compounds present in the *P. anisum* seed aqueous extract was performed using the aqueous extract to identify active ingredients such as phenolic compounds, tannins, alkaloids, flavonoids, carbohydrates, proteins, terpenoids, steroids and saponins using standard procedures [25,26].

2.4 Synthesis of Silver Nanoparticles

An aliquot of 10 ml of the *P. anisum* seed aqueous extract was slowly added to 90 ml of 2mM $AgNO_3$ solution in the Erlenmeyer flask under continuous stirring using Torrey Pines Scientific magnetic stirrer, USA.

2.5 Characterization of Synthesized AgNPs

2.5.1 UV-visible spectroscopy

UV-Vis absorption spectra of the reaction mixture were recorded in the wavelength range of 200 to 800 nm by taking a small aliquot of the reaction mixture in a quartz cuvette to examine the surface plasma resonance (SPR) using a UV-Visible spectrophotometer (V-530, Japan Servoco. LTD. Indonesia) [27].

2.5.2 Fourier transforms infrared spectroscopy

Green synthesized AgNPs and *P. anisum* seed aqueous extract were analyzed by FTIR (Spectrum Nicolet IS 10, Thermo scientific, USA). The bands were used to characterize different functional groups involved in the reduction of silver ions and capping of AgNPs by bioactive compounds, present in *P. anisum* seeds. FTIR was operated at wave number with a range of 500 - 4000 cm^{-1} [28].

2.5.3 High resolution electronic microscopy analysis

Morphology and size of the synthesized AgNPs were tested using High Resolution Electronic Microscopy (HR-TEM) (Tecnai G 20, FEI Europe, Eindhoven, The Netherlands) with an accelerating voltage of 200 kV and selected area electron diffraction (SAED) pattern (Jeol, JSM-5800, Osaka, Japan) was used to detect the formation of AgNPs. A drop of biosynthesized AgNPs solution (dissolved in distilled water) was kept on a carbon-coated copper grid, then dried at room temperature and, finally, transferred to the microscope or analysis [29].

2.6 Antibacterial Activity of AgNPs

The *in vitro* antibacterial activity of the synthesized AgNPs against five isolates of plant pathogenic bacteria in addition to *P. tolaasii* was carried out using the agar well diffusion method [30]. The overnight culture diluted in 10 mM phosphate buffer (2.7g $Na_2HPO_4.12H_2O$; 0.4g $NaH_2PO_4.2H_2O$; distilled water to 1.0 liter and final pH 7.2) until the optical density (at λ=600 nm) is between 0.07 and 0.1using UV/Visible spectrophotometer (MODEL:2000 UV- UNICO INSTRUMENTS CO., LTD,USA). This is roughly equivalent to 1×10^8 CFU/ml [31]. Each flask of 250 ml nutrient glucose agar (NGA) media was inoculated individually (after sterilization and cooled to 50°C in a water bath) with 3 ml of one of bacterial suspensions (10^8 CFU/ml). Petri plates were prepared by pouring 20 ml/plate (12 plates for each isolate) of NGA for each of the tested bacteria. Once the agar was solidified, plates were punched using a sterile cork borer (6 mm diameter). The experiment was performed in three replicates (plates). Four wells were made in each Petri plate, three for the tested material, and one for the control treatment. Each well was filled with 40 µl of the prepared AgNPs, kept 30 min at room temperature for diffusion, and then incubated at 28°C for 48 h. The inhibition zones formed around the wells were measured to the nearest millimeter with a calibrated ruler. The same procedure was repeated for an aqueous solution of 2mM of $AgNO_3$, and *P. anisum* seed aqueous extract.

2.7 Statistical Analysis

A completely randomized design was used in the well diffusion sensitivity assay; zones of inhibition were measured to the nearest millimeters with a calibrated ruler. Data are expressed as the mean value of three independent replicates. Statistical analysis was carried out by one-way analysis of variance (ANOVA) test followed by Duncan's multiple range tests [32] at a 5% level of significance ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Characterization of Silver Nanoparticles

Biosynthesis of NPs is an important field in nanotechnology, which does not necessarily use

high pressure, energy, temperature, toxic chemicals and is eco-friendly. In addition; it would be suitable for developing a biological process for large-scale production [33-36].

The formation of AgNPs using *P. anisum* seed aqueous extract is observed at room temperature within 24 h through the color change of the reaction mixture from colorless to dark brown. This color change is attributed to the excitation of surface plasmon resonance and reduced silver ions by the seed extract (Fig. 1), in accordance with [28]. The UV-vis absorption spectra of AgNPs showed a strong absorption band at 441 nm, as clear in Fig. 2. these results of the UV-vis spectroscopy are consistent with [37] suggested a peak plasmon resonance (SPR) between 410-450 nm due to the formation of AgNPs.

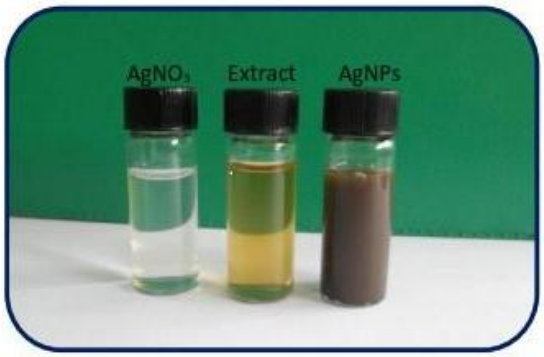

Fig. 1. Visual observation of color change due to the formation of AgNPs

3.2 High Resolution Electronic Microscopy Analysis

Results of the High-Resolution Electronic Microscopy (HR-TEM) image of the synthesized AgNPs demonstrated it is spherical and its size ranged from 15- 37 nm (Fig. 3). According to [38,39], the formation of different shapes and sizes of the NPs might be attributed to the presence of more than one reducing agent in the plant extract. Fig. 4 showed the selected area electron diffraction pattern (SAED) of the silver nanoparticles, the ring patterns indicate that the particles are crystalline [40].

3.3 FT-IR Spectra

FTIR spectrum studies are used to identify the possible biochemical groups responsible for reducing the silver ions and act as capping agents to achieve the effective stability of the AgNPs [41,42]. The dual roles of the seed extract as a reducing and stabilizing or capping agent is recognized by comparing the spectrum of the *P. anisum* seed extract (Fig. 5a) and the synthesized AgNPs (Fig. 5b). These figures showed a shift in the following peaks at 3419.7-3432.2, 2925.2-2924.8, 1633.6-1626.9, and 1035.1-1076.5 cm^{-1}. The peak at 3419.7 cm^{-1} observed in the seed extract is attributed to the -OH group stretching vibration and is shifted to higher frequency regions at 3432.2 cm^{-1}. The hydroxyl groups (OH) present in the polyphenols and alcohol is detected in the seed extract and

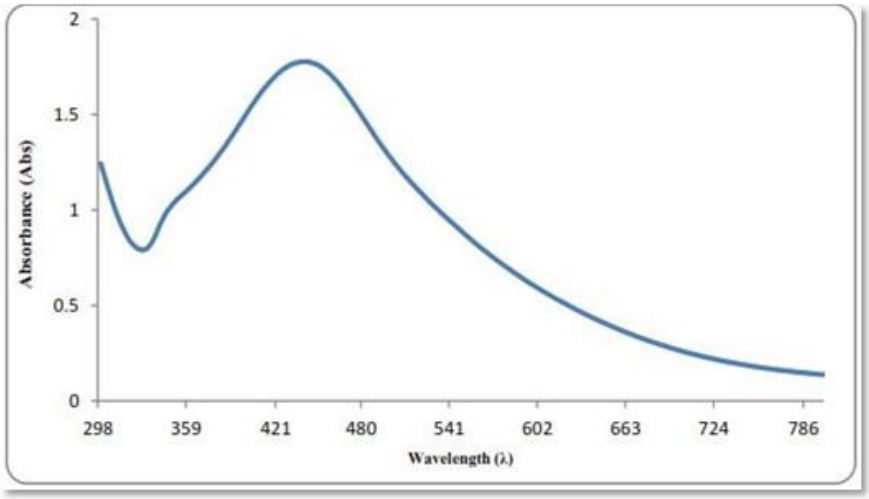

Fig. 2. UV-vis spectrum of synthesized AgNPs from *P. anisum* seed aqueous extract

may be responsible for the bio-reduction process during AgNPs synthesis, as reported [43]. The band observed at 2925.25-2924.84 is attributed to the C-H stretching vibration of alkenes [44]. The band observed at 1633.68 cm^{-1} in the extract is due to amide I vibrations. In accordance with [45], this band is shifted to 1626.96 cm^{-1} in the AgNPs because of the possible presence of proteins that will bind to AgNPs through the amine groups. Meanwhile, the band shifted at 1626.96 cm^{-1} is assigned to the C=O functional group with AgNPs. The observed peaks are mainly attributed to flavonoids and terpenoids present in plant extract [38]. The band detected at 1035.03-1076.54 cm^{-1} is attributed to C-N stretching vibration, possibly due to the presence of aliphatic amines that are commonly found in proteins.

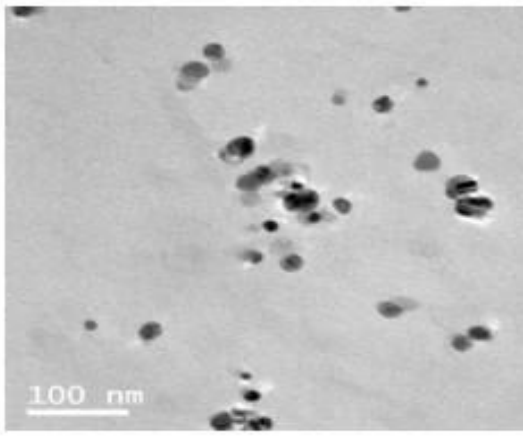

Fig. 3. HR-TEM image of synthesized AgNPs from *P. anisum* seed aqueous extract

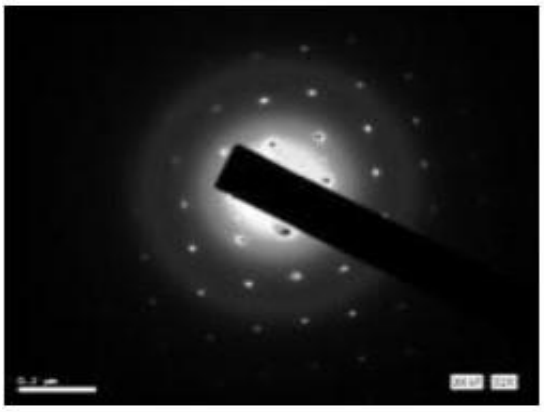

Fig. 4. Selected area electron diffraction (SAED) image of synthesized AgNPs from *P. anisum* seed aqueous extract

3.4 Phytochemical Analysis

Phytochemical analysis of the *P. anisum* seed aqueous extract revealed the presence of carbohydrates, and common secondary metabolites including phenolic compounds, flavonoids, terpenoids, saponins, and tannins as shown in Table 1. It can be stated that the hydroxyl and carbonyl groups present in carbohydrates, flavonoids, terpenoids, and phenolic compounds, can play major roles in the reduction of Ag^{+} to Ag^{0} [46,47].

Table 1. Preliminary phytochemical analysis of *P. anisum* seed aqueous extract

Test	Constituents	Screaming
1	Carbohydrates	+
2	Phenolic compounds	+
3	Flavonoids	+
4	Terpenoids	+
5	Saponins	+
6	Tannins	+
7	Alkaloids	-
8	Steroids	-

+ Present of the chemical compound, -absence of the chemical compound

3.5 Antibacterial activity of AgNPs

The use of biogenic AgNPs in the protection of crop diseases offers an excellent promise in the management of insects and microbial pathogens, as an alternative to the chemically used pesticides. According to [48]. AgNPs are remarkably effective against several phytopathogens with lower phytotoxicity and have broad spectrum of activities including acting as; pesticidal, antiviral, antifungal, antibacterial as well as nematicidal. AgNPs can be used as a foliar spray to halt the growth of mold fungi, bacteria, and viruses, due to its quite stability and of being highly dispersive in water.

The *in vitro* antibacterial activity of the biosynthesized AgNPs, $AgNO_3$, *P. anisum* seed aqueous extract and de-ionized water was studied against some important phytopathogenic bacteria as shown in Table 2. The AgNPs showed higher antibacterial activity compared to the ionic silver. The AgNPs recording maximum diameter of inhibition zones about (30.0 mm) for *E.amylovora*, which is followed by *A.tumefaciens* (25.0 mm), *R. solanacearum* (20.0 mm), *P. tolaasii* (19.0 mm), *P. lachrymans* (15.0 mm),

and finally *E. carotovora* subsp. *Carotovora* (10.0 mm), as clear in Fig. 6. Meanwhile, the control treatment (de-ionized water) and the seed extract did not exhibit any inhibition zones. The antibacterial effect of the biogenic AgNPs may be attributed to its small size, thus becomes able to reach the nuclear material of the bacteria easily. Also, AgNPs has greater surface area, better contact and interaction with the bacterial cell, in accordance with the previous findings of [44,49,50]. Thus, from all the above-mentioned results, it is evident that AgNPs may have an important agriculture application in controlling the phytopathogenic bacteria.

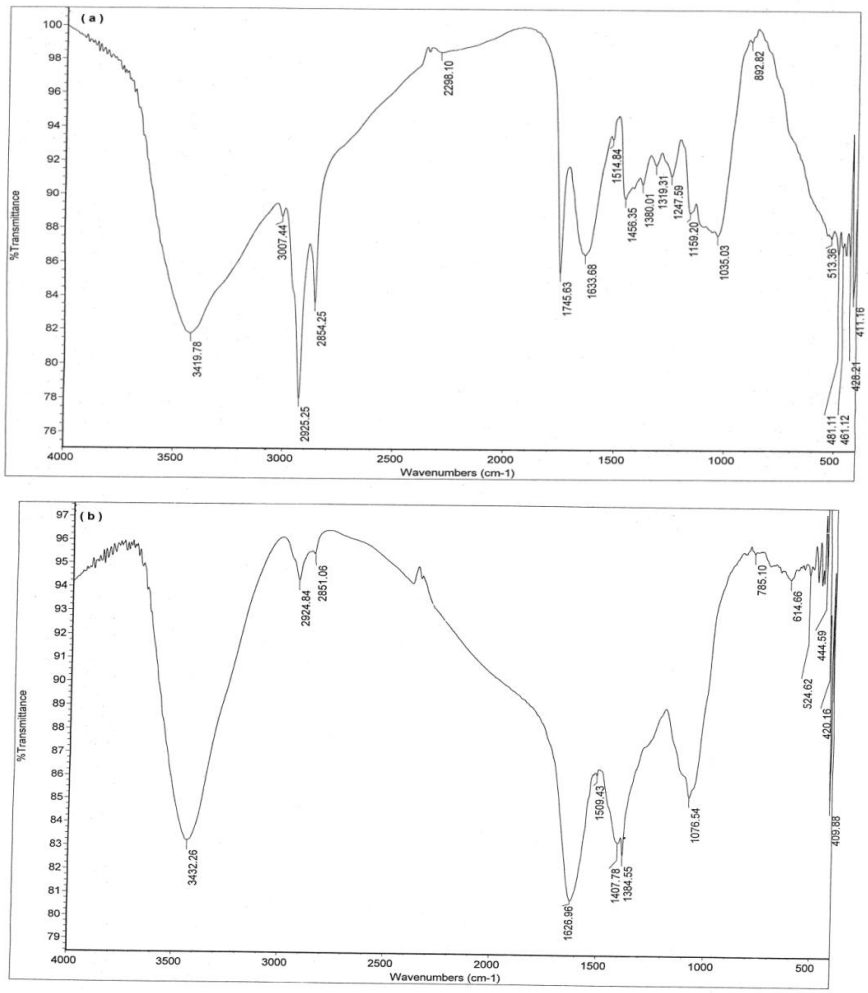

Fig. 5 (a-b). FTIR spectrum of the *P. anisum* seeds extract. (a) and the synthesized AgNPs (b)

Table 2. Antibacterial activity of AgNPs synthesized from *P. anisum* aqueous seeds extract

Tested materials	Zone of inhibition (mm)*					
	A. tumefaciens	***E. amylovora***	***E. carotovora* subsp.*carotovora***	***P. lachrymans***	***Ralstonia solanacearum***	***P. tolaasii***
$AgNO_3$**	10.0[b]	15.0[b]	6.0[b]	7.0[b]	12.0[b]	13.0[b]
AgNPs***	25.0[a]	30.0[a]	10.0[a]	15.0[a]	20.0[a]	19.0[a]
Seed extract	6.0[c]	6.0[c]	6.0[b]	6.0[b]	6.0[c]	6.0[c]
Control	6.0[c]	6.0[c]	6.0[b]	6.0[b]	6.0[c]	6.0[c]

Means of three replicates; Means shared a letter in the same column are not significantly different using Duncan's Multiple Range Test ($p \leq 0.05$)
*Where; * including well diameter (6 mm), ** Aqueous solution of 2mM of $AgNO_3$; *** Biogenic AgNPs*

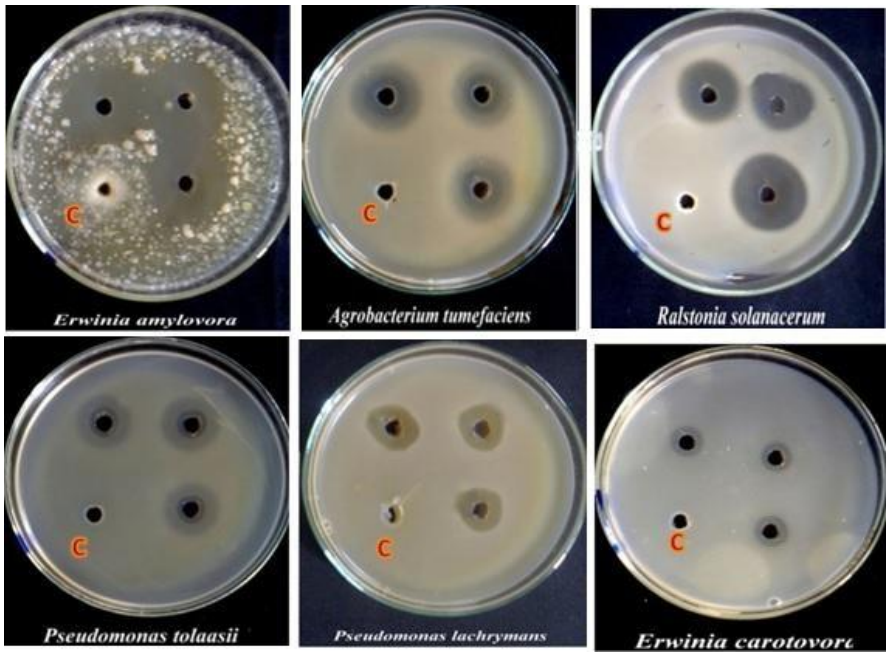

Fig. 6. Antibacterial activity of synthesized AgNPs against some phytopathogenic bacteria, and *P. tolaasii*, showing clear inhibition zones of different diameters.
Where, C = Sterile de-ionized water

4. CONCLUSION

In this study, an aqueous extract of *P. anisum* seeds is used to synthesize AgNPs, where the seed extract acted as a reducing agent for NPs synthesis. This method of green synthesis of AgNPs is simple, rapid, and eco-friendly. The biosynthesized AgNPs had spherical shapes with particle size ranging from 15- 37 nm, crystalline in nature, and showed UV-vis absorption spectrum at 441 nm. This AgNPs demonstrated *in vitro* antibacterial efficacy against several phytopathogenic bacteria. Future research is recommended to test the *in vivo* effects of AgNPs on the crop plants growing under greenhouse and field conditions.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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