GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Influence of abiotic conditions on synthesis of silver nanoparticles using *Verbascum scamandri*

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GSC Biological and Pharmaceutical Sciences, 2025, 30(02), 252-260

Publication history: Received on 06 January 2025; revised on 15 February 2025; accepted on 18 February 2025

Article DOI: https://doi.org/10.30574/gscbps.2025.30.2.0064

Abstract

Green synthesis involves the biological production of metallic nanoparticles using plants or plant extracts. In our study, we investigated the effect of altitude on synthesizing silver nanoparticles (AgNPs) from *Verbascum scamandri* plants collected at two distinct altitudes in Bayramiç, Çanakkale, Türkiye (*V1*: 675 m, *V2*: 800 m) and from *in vitro* grown samples (*V3*), using a green synthesis method. UV-Vis spectroscopy confirmed the formation of AgNPs with peak absorption between 428-432 nm. Zeta potential analysis showed that AgNPs had negative surface charges, indicating stability. The sizes of nanoparticles varied significantly, with *V3-AgNPs* exhibiting the largest average size of 242.7 nm, and *V2-AgNPs* the smallest at 125.1 nm. FTIR analysis revealed functional groups responsible for reduction, including -OH, -CN, and C=O bonds. SEM and TEM analyses confirmed spherical AgNPs with sizes ranging from 20-97 nm. EDX analysis indicated high Ag content, with *V3-AgNPs* showing 87.79% Ag. The antimicrobial activity of AgNPs was assessed against various bacterial and fungal strains. The minimum inhibition concentrations (MIC) ranged from 0.62 to 5.0 µg/mL, with *V3-AgNPs* showing the highest activity against *Acinetobacter baumanii* (MIC = 0.62 µg/mL). The biofilm inhibition percentage varied, with *V3-AgNPs* demonstrating the highest inhibition against *A. baumanii* (82.05%) and *Escherichia coli* (76.12%). Altitude and environmental factors influenced AgNP synthesis, with the highest particle sizes from lower altitudes and roadside samples.

Keywords: Altitude; Green Synthesis; Antibiofilm Activity; Mullein; Nanoparticle Stability

1. Introduction

Nanotechnology increasingly utilizes natural resources for synthesizing nanoparticles (NPs). Green synthesis methods, which use plants extracts, are more environmentally and allow for better control over particle size and shape [1]. Silver nanoparticles (AgNPs), a key product in this field, are generally non-toxic in low quantities and are used in textiles, food packaging, medicine, cosmetics, and more. Plant extracts can serve as capping and reducing agents for AgNP production, reducing the need for harmful chemicals. They contain polyphenols and other secondary metabolites that facilitate nanoparticle formation [2].

Verbascum scamandri Murb., known as Kazdağı Sığırkuyruğu, is a biennial endemic to Türkiye, reaching about 80 cm in height with a smooth, cylindrical stem. It typically grows in *Pinus nigra* forests. This species contains various secondary metabolites including flavonoids [3], alkaloids [4], essential oils [5], neolignan glycosides and terpenes [6], phenolic, iridoids, and phenylethanoids [7], which contribute to its recognized antimicrobial [8-9], anticancer [10], antioxidant [11], or anti-inflammatory effects [12].

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Secondary metabolites are essential compounds produced by plants for defense and adaptation against various abiotic (e.g., temperature, humidity) and biotic (e.g., bacteria, insects) stresses. They significantly influence plant-environment interactions and have positive effects on human health, particularly in pharmaceuticals and food. The quantity of these metabolites varies based on environmental factors like pH, soil type, temperature, altitude, and water availability, with altitude notably impacting their synthesis [13].

This study aims to investigate whether the structural properties and biological activities of silver nanoparticles (AgNPs) synthesized from *V. scamandri* plant samples, which were collected from different altitudes and exposed to various abiotic factors, exhibit any significant variations. Specifically, we seek to determine how altitude and other environmental conditions influence the formation, size, shape, and potential bioactivity of the AgNPs produced through the green synthesis method.

2. Materials and Methods

2.1. Plant Materials

Plant specimens of *V. scamandri* were gathered from diverse locations and altitudes in Bayramiç, Çanakkale, Türkiye, during the flowering season in August and September of 2021 (Table 1 and Figure 1a).

Table 1 The locations of the plant samples of *V. scamandri* collected from the field

No	Locality	Altitude (m)	Latitude Longitude	Period of Collection	Location features
V1	Çırpılar Village, Bayramiç	675	39°47'03.2" N 26°53'57.5" E	August	Roadside
V2	Evciler Village, Bayramiç	800	39°45'19.7" N 26°51'08.2" E	September	Wooded area

After collecting, the field-grown plant samples (*V1* and *V2*) were washed with distilled water to remove any dust or impurities and subsequently air-dried at room temperature (20 - 25°C). Seeds of *V. scamandri* were also obtained from Çanakkale-Bayramiç, Türkiye. The *in vitro* germination of seeds (*V3*) was carried out according to the study conducted by Cambaz and Çördük [14]. All plants were cultivated in a growth chamber at 25±2 ℃, subjected to a 16-h light/8-h dark photoperiod, with a light intensity of 72 µmol $m^{-2}s^{-1}$ and 50±5% humidity. 11-week-old, *in vitro* grown plant samples (*V3*) were used for the analyses (Figure 1b).

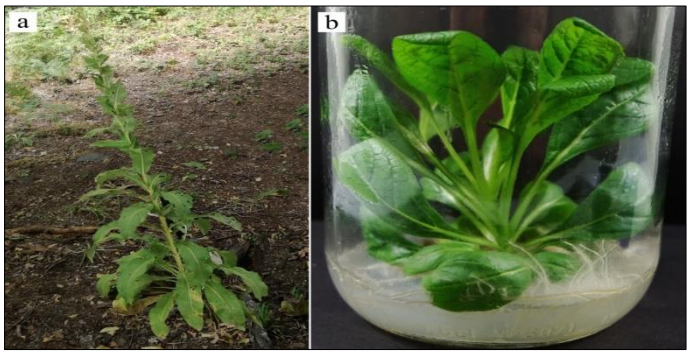

Figure 1 (a) The plant sample of *V. scamandri,* (b) 11-week-old *in vitro* grown plant (*V3*) of *V. scamandri*

2.2. Synthesis and Characterization of AgNPs

V. scamandri leaves were air dried and crushed (5 g), then extracted with 100 mL of sterile distilled water at 65–70°C for 60 min. For AgNP production, an aqueous solution of $AgNO_3$ (1 mM) and *V. scamandri* extract (100 mL) was mixed at a ratio of 1:9 and allowed to react in a bottle at room temperature. The dark solution was centrifuged at 8.000 rpm for 5 min. The pellet (AgNP) was dried in the oven at 50 ℃ for 48 hours [15] and collected in Eppendorf's.

A UV-visible (UV-Vis) absorption spectrophotometer (Shimadzu) with a resolution of 1 nm between 200 and 800 nm was utilized. One milliliter of the material was pipetted into a test tube and then examined at room temperature. The average size of the produced AgNPs was determined using dynamic light scattering [16].

Size distributions and surface charges of the synthesized AgNPs were measured with the Zetasizer device [17]. Measurements were taken at 173 ° angle and 25 °C temperature for particle size and at 12° angle and 25 °C temperature for zeta load for three times.

FTIR analysis to determine the functional groups responsible for reduction contained in AgNPs; it was made in the range of 4000-400 cm^{-1} using the Perkin Elmer BX II FT spectrometer device in Atatürk University Eastern Anatolia High Technology Application and Research Center (DAYTAM).

AgNPs were determined using Scanning Electron Microscope (SEM) [(Quanta FEG 250 (FEI, USA)], and Transmission Electron Microscope (TEM) (Hitachi HT-7700). EDX (LEO 1430 VP) was used to elucidate the elemental composition of the synthesized NPs [18]. The analysis was carried out as a service by DAYTAM.

2.3. Antagonistic Activities of AgNPs

Antimicrobial effects of AgNPs were determined by using the micro dilution method [19], Minimum Inhibition Concentration (MIC), and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) using *Staphylococcus haemolyticus* ATCC 43252, *Acinetobacter baumanii* ATCC 19606, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* NRRL B-3704, *Bacillus subtilis* ATCC 6633, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 27853 strains and *Candida albicans* ATCC 10231 yeast.

The microplate biofilm method was used to evaluate the inhibition of biofilm formation by *V. scamandri - AgNP* (*V-AgNP*) plant extracts against test organisms [20]. The measurement of the antibiotic effect of the extracts was made by the percentage reduction formulation.

$$\% \text{ Inhibition} = (A_{control} - A_{sample} / A_{control}) \times 100$$

$A_{control}$: Absorbance of the control [containing 100 µL Mueller Hinton Broth (MHB) instead of plant extract] reaction; A_{sample}: Absorbance of the test compounds

3. Results

3.1. UV-Vis Spectrophotometer

During the formation of AgNPs, the change of the solution from the original yellow color to burgundy-brown after the addition of $AgNO_3$ to the extract confirms the existence of a redox reaction. A strong peak between 428-432 nm was observed in the absorption spectrum, indicating the formation of AgNPs (Figure 2). This band corresponds to the surface plasmon resonance of the NPs, confirming their formation. In the Uv-Vis analysis, the highest absorbance value was obtained from *V2-AgNP* (A = 0.12).

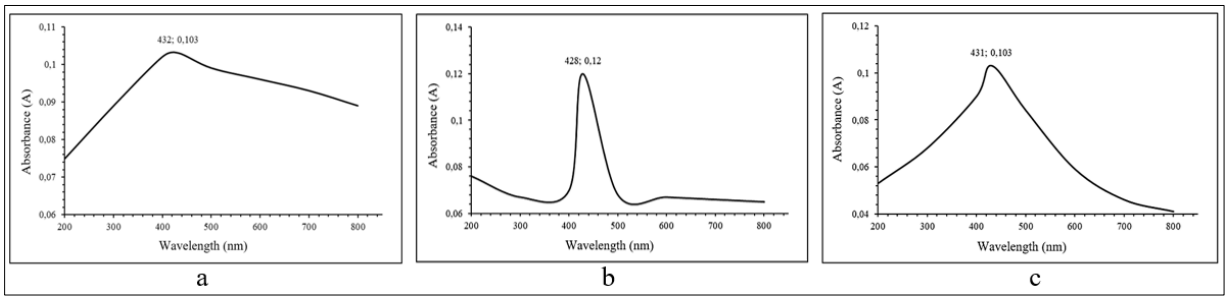

Figure 2 UV-Vis spectra of a) *V1-AgNP*, b) *V2-AgNP*, c) *V3-AgNP*

3.2. Zeta Potential Analysis

All AgNPs exhibit a negative charge, indicating the absence of any aggregation or precipitation and confirming their stability. Zeta values of synthesized AgNPs were found to be; *V1* particle size 163.6±3.593 nm, polydispersity index 0.45, zeta potential charge -18 mV; *V2* particle size is 125.1±5.829 nm, polydispersity index is 0.579, zeta potential charge is -20.9±0.265 mV, and *V3* particle size is 242.7±2.495 nm, polydispersity index is 0.565, zeta potential charge is -20.7± 0.503 mV (Figure 3).

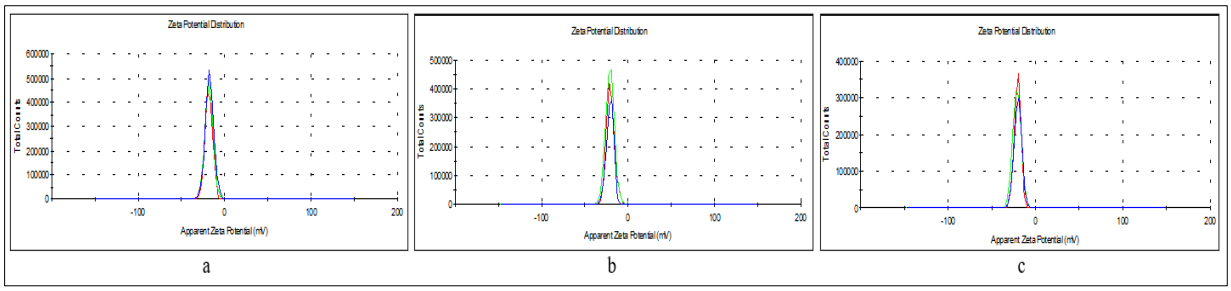

Figure 3 Surface charge of the colloidal system of a) *V1-AgNP,* b) *V2-AgNP,* c) *V3-AgNP*

3.3. FT-IR Analysis

Phytochemicals and functional groups responsible for the reduction were identified by FT-IR spectroscopy (Figure 4). In comparison, it is seen that the prominent peaks involved in the reduction belong to -OH, C=O, and -C-C bonds. In the synthesis of AgNPs, it has been determined that OH, -CN, and C=O groups play a role in reduction, with frequency shifts occurring in the spectra between 3200 cm^{-1} and 3800 cm^{-1}. It characterizes the OH groups of the 3269 cm^{-1} and 3464 cm^{-1} spectrums. It is known that the peaks between 2245 cm^{-1} and 2644 cm^{-1} belong to -CN stretches, and the peaks at 1018 cm^{-1}, 1444 cm^{-1} and 1651 cm^{-1} belong to C=O and C=C stretches. The absorption peak at 1867 cm^{-1} can be identified as amide I (Figure 4a-c).

Fluctuations in the range of 1200 cm^{-1}- 1600 cm^{-1} peaks in the *V2-AgNP*, and *V3-AgNP* FTIR graphs may be due to the characteristic features of flavonoids/phenolic groups. The downward peaks seen in *V1-AgNP* in these ranges indicate changes in phenolic groups. In the FTIR graph, there was a decrease in the peaks of *V2-AgNP* at 3275 cm^{-1}. This is thought to be due to the *V2* plant moving away from sea level, not being able to provide optimum conditions for secondary metabolite production, or the direction factors of the region where it is collected are effective on the amount of secondary metabolites such as flavonoids and terpenes.

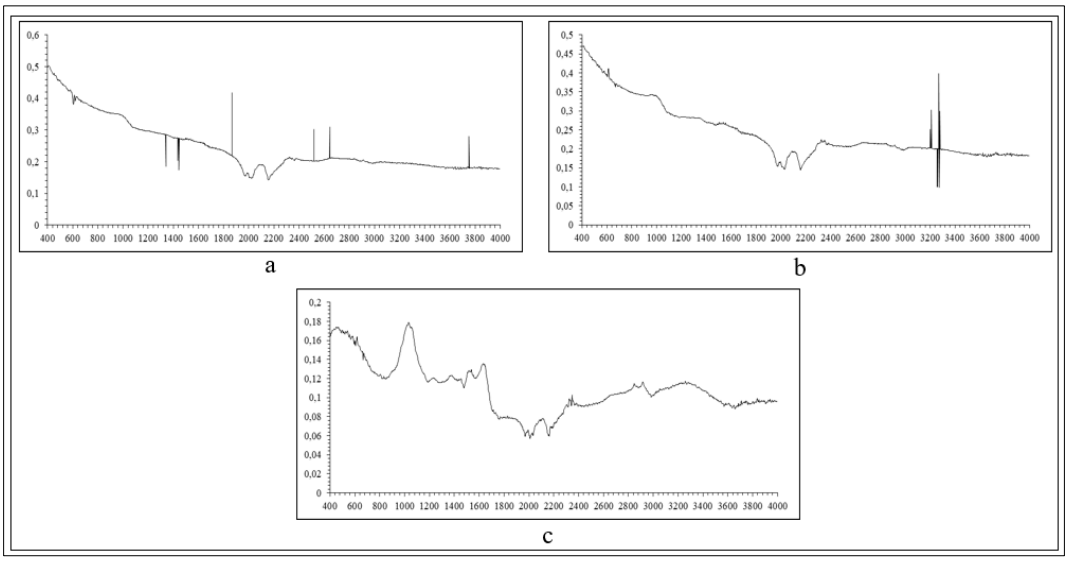

Figure 4 FTIR analysis of a) *V1-AgNPs*, b) *V2-AgNP*, c) *V3-AgNP*

3.4. SEM, TEM, and EDX Analysis

SEM is a crucial technique for analyzing the size, shape, and surface morphology of nanoparticles. In this study, AgNPs were randomly dispersed on a plant platform, with sizes ranging from 51 to 97 nm (Figure 5).

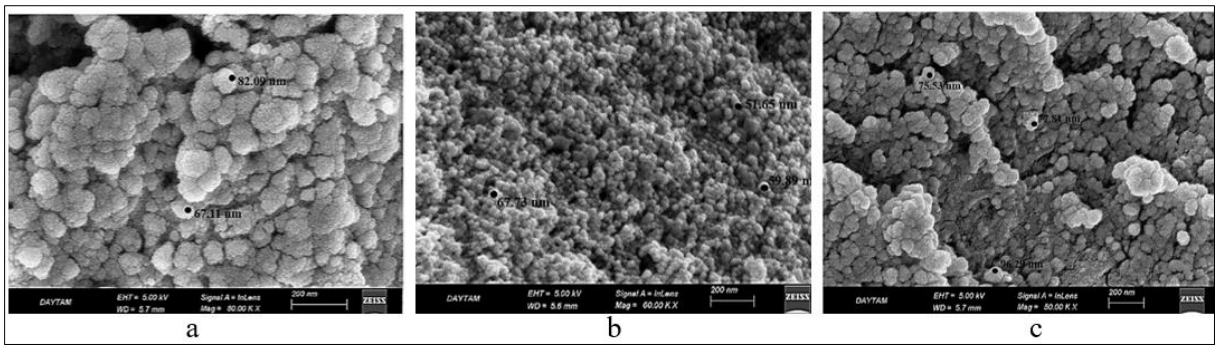

Figure 5 SEM images of a) *V1-AgNPs,* b) *V2-AgNP,* c) *V3-AgNP*

AgNPs were examined using a field emission TEM device and it was determined that the obtained AgNPs were spherical in the size range of 20-46 nm (Figure 6).

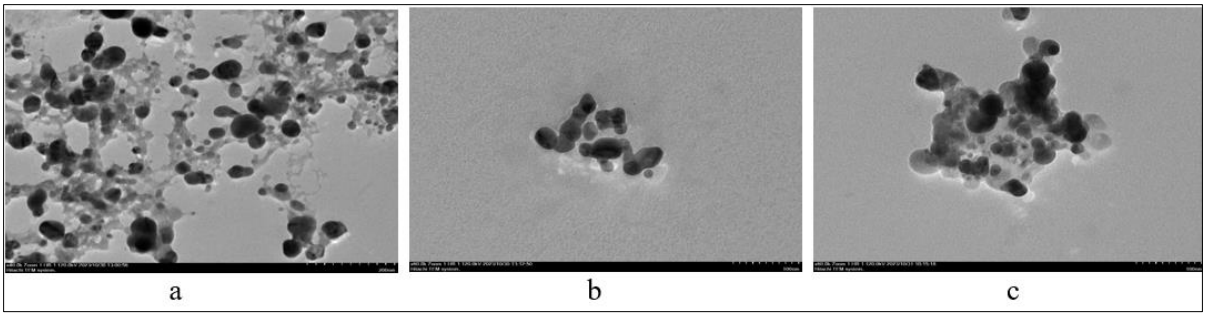

Figure 6 SEM images of a) *V1-AgNPs*, b) *V2-AgNP*, c) *V3-AgNP*

The purity and elemental composition of the NPs were determined using EDX and elemental mapping. The EDX spectrum showed an optical absorption band peak at 3 keV, corresponding to the surface plasmon resonance (Figure 7). NPs exhibit excellent dispersion in bio-reduced aqueous solutions, even on a macroscopic scale. These findings have proven that the synthesized product is AgNPs. The ratios of Ag and O in *V1, V2,* and *V3- AgNPs* were determined to be 82.79% Ag and 17.21% O, 72.24% Ag and 27.76% O, 87.79% Ag, and 12.21% O, respectively.

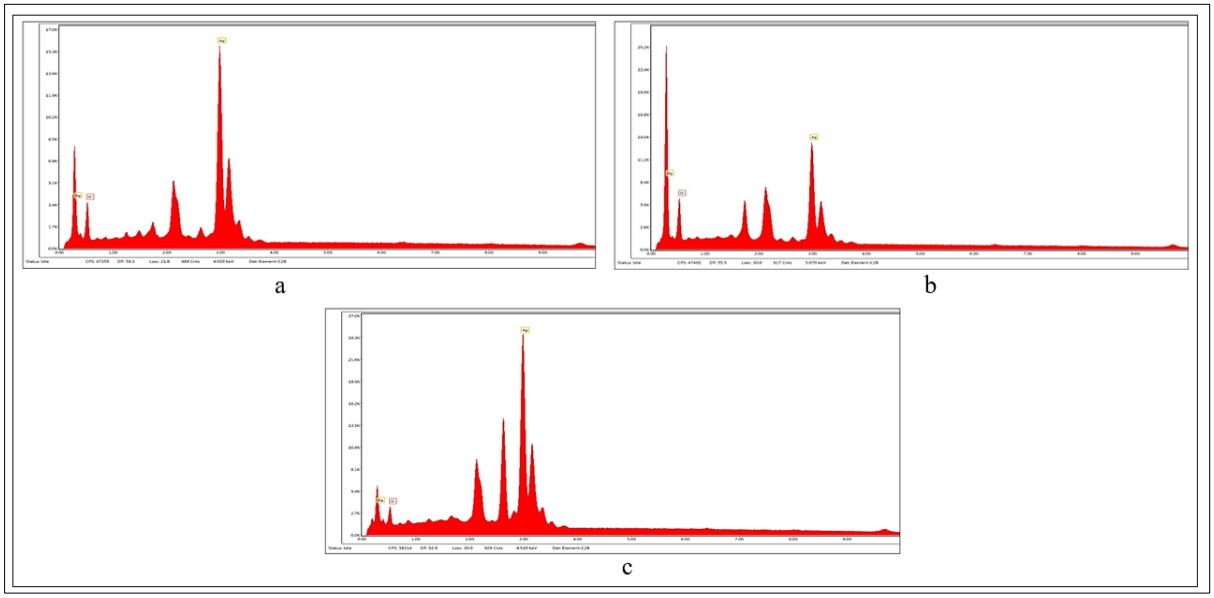

Figure 7 EDX analysis of of a) *V1-AgNPs*, b) *V2-AgNP*, c) *V3-AgNP*

3.5. Antimicrobial Activities of AgNPs

The MIC and MBC findings of the aqueous extracts are summarized in Table 2. In gr (-) bacteria, the MIC values of *AgNPs* range between 0.62 - 5.0 µg/mL. Higher MIC values were obtained from V3 against *A. baumanii* and *V1* and *V2* against *E. coli*, compared to the control antibiotic. MIC values of AgNPs in gr (+) bacteria were determined between 1.25 - 5.0 µg/mL. It was found to be higher the MIC values obtained from *V2*, and *V3-AgNP* against *B. subtilis*. MIC values of NPs for *C. albicans* were generally detected between 2.5 - 5.0 µg/mL. The MBC/MFC values of *V-AgNPs* varied between 2.5 - 5 µg/mL (Table 2).

Table 2 Antimicrobial activities of AgNPs

Test Cultures	**MIC**			**Control antibiotics**		**MBC or MFC (µg/mL)**		
	AgNPs					**AgNPs**		
	V1	**V2**	**V3**	**S10**	**NY100**	**V1**	**V2**	**V3**
Gr (-)								
A. baumanii	5.0	2.5	0.62	2.0	NT	5.0	5.0	2.5
E. coli	1.25	2.5	1.25	4.0	NT	2.5	5.0	5.0
P. vulgaris	5.0	5.0	5.0	4.0	NT	5.0	5.0	5.0
P. aeruginosa	5.0	5.0	5.0	1.0	NT	5.0	5.0	5.0
Gr (+)								
S. haemolyticus	5.0	5.0	5.0	5.0	NT	5.0	5.0	5.0
S. aureus	5.0	5.0	5.0	4.0	NT	5.0	5.0	5.0
B. subtilis	5.0	2.5	1.25	4.0	NT	5.0	5.0	5.0
Yeast								
C. albicans	5.0	5.0	2.5	NT	2.5	5.0	5.0	5.0

S10: Streptomycin (10 µg/disc); NY100: Nystatin (100 µg/disc); NT: No Tested

The highest biofilm percentage was obtained from *V3-AgNP* extract against *A. baumanii* and *E. coli* (Table 3). Furthermore, significant antibiofilm activity has been observed against *B. subtilis* (*V3-AgNP*), *A. baumannii* (*V2-AgNP*), and *E. coli* (*V1-AgNP*, *V2-AgNP*) strains.

Table 3 Antibiofilm (% Inhibition) activities of *V-AgNPs* extracts

Extracts	**Concentration**	**Test Cultures**							
		A.baumanii	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*S. haemolyticus*	*S. aureus*	*B. subtilis*	*C. albicans*
V1	MIC	29.13±1.1	71.65±0.5	28.05±0.2	36.15±1.1	26.15±1.1	31.2±0.1	35.12± 0.1	31.01±1.1
	MIC/2	-	52.12±0.5	-	18.15±0.1	-	12.01±0.1	-	-
V2	MIC	61.01±1.1	53.15±1.1	33.45±0.14	34.01±0.3	34.13±1.1	30.45±1.14	44.22±1.21	36.01±1.1
	MIC/2	31.01±1.1	22.02±0.2	-	28.15±1.1	-	12.13±1.1	14.12±1.2	-
V3	MIC	82.05±1.1	76.12±0.1	28.12±1.1	36.04±0.5	37.11±0.1	32.25±0.12	67.03±0.1	38.01±1.1
	MIC/2	68.04±0.2	48.02±0.1	-	-	22.01±0.1	-	43.12±0.1	

4. Discussion

In the study, AgNP was synthesized from samples of the *V. scamandri* plant collected from two different locations and grown under *in vitro* conditions. Our research is the first study to investigate NP synthesis from *V. scamandri*, a locally endemic plant species, and its antagonistic activities, furthermore, examining different locations and *in vitro* production. The largest particle size was obtained from *V3*, and the smallest particle size was obtained from the samples collected from the *V2*. TEM analysis results were determined that all AgNPs were spherical in size. In EDX analysis, the highest percentage of Ag content was found to be *V3-AgNP* (87.79%). The lowest Ag content was determined as *V2-AgNP* (72.24%). AgNP synthesis was proven by the absorbance values giving the maximum peak obtained in the UV-Vis analysis, all AgNPs were determined to be negatively charged. In general, the presence of negative charges on the surfaces of NPs affects the properties of the surrounding liquid and the behavior of the nanoparticles. This can determine the distribution, aggregation, or interactions of NPs in solution. FTIR analysis was performed to determine the functional groups of the produced AgNPs. It is thought that the fluctuations in the characteristic peaks representing phenolic groups in the produced *V2-AgNP* affect the nanoparticle size and polydispersity. These findings are consistent with previous research on the characterization properties of greenly synthesized AgNPs by Badola and Negi [21], Aboutorabi et al. [22], and Gonca et al. [23].

Both the field-grown and *in vitro* grown plant samples NPs have higher antibacterial and antibiofilm activities against gram (-) than gram (+) bacteria. Furthermore, AgNPs synthesized from the plant extract outperform the plant extract alone in terms of antagonistic activity [24]. These findings are in contrast with previous research on the antimicrobial properties of greenly synthesized AgNPs by Badola and Negi [21], Geyesa et al. [25]. Additionally, some researchers have similarly detected high antibacterial [26] and antibiofilm [23] activities against gram (-) bacteria, consistent with our findings. It is believed that the variation in antagonistic activities among test cultures is associated with strain differences and different secondary metabolite contents in various *Verbascum* species.

Secondary metabolites do not directly play a role in the growth and development of plants, but they are crucial compounds for adaptation and defense under stress conditions. The effects of both biotic and abiotic environmental factors can also vary with altitude. For instance, the density and diversity of plant pollinators may decrease with altitude. Additionally, it is assumed that as one move from low to high altitudes, the number and diversity of herbivores decrease, along with a reduction in anti-herbivore compounds [24]. It is known that during plant growth and development, the production of secondary metabolites is influenced by abiotic conditions as well [27], thereby affecting the quality of plant raw materials. For instance, in a study on *Hedychium spicatum* [28], it was indicated that the phenolic content increased with increasing altitude. Similarly, differences in secondary metabolite content at different altitudes have been reported in *Primula denticulate* species [29].

According to the results obtained in our study, larger-sized NPs were synthesized from samples of *V. scamandri* collected from different altitudes (675 m) and roadside areas in August (*V1*). In September, the sample collected from a forested area at a higher altitude (800 m) yielded the smallest NP size. When compared to the *in vitro* grown sample (*V3*) unaffected by seasonal and other abiotic stress conditions, it was determined that *V3* had the largest NP size than other synthesized NP. In most studies investigating the effect of altitude on secondary metabolite synthesis, an increase in altitude has been reported to correspond with an increase in secondary metabolites [30]. However, a few studies have suggested that secondary metabolites may decrease with increasing altitude. For instance, on *Origanum majorana* species, it was observed that the ratio of metabolites decreased with an increasing altitude. The flowering period of the plant was reported to have the highest essential oil content at the lowest altitude of 766 m. Plants collected from different altitudes (766 m, 890 m, 968 m, 1079 m, 1180 m, 1261 m, and 1387 m) showed that the essential oil ratio decreased with increasing altitude [31]. A study conducted on *Agriophyllum squarrosum* species similarly indicated a decrease in flavonoid content with increasing altitude [32]. In this context, contrary to expectations, it is believed that the *V. scamandri* plant samples, due to their presence in roadside or forested areas with increasing altitude, reduced secondary metabolite synthesis and consequently decreased NP synthesis.

5. Conclusion

In conclusion, in our study investigating the effect of altitude on nanotechnological methods for NP synthesis from plants, the most effective results were obtained from the sample collected at the lowest altitude and roadside area (*V2*) and the *in vitro* grown sample (*V3*). However, to prevent harm to plant populations and the risk of extinction, it is recommended to use *in vitro* grown plants (*V3*) due to their relatively similar antimicrobial properties and NP size characteristics, as well as ease of production.

This article examines the effects of factors such as temperature changes, pressure differences, and plant location on the secondary metabolites and nanoparticle synthesis produced by plants when plants are exposed to various factors, especially at high altitudes. The study also forms the basis for future studies on the effects of secondary metabolites produced by plants when under stress on NP synthesis, and how the stress factors that plants are exposed to affect NP synthesis by increasing or decreasing secondary metabolite production. This research will also provide the basis for elucidating the complex relationship between the stress factors that plants encounter in their natural environment, secondary metabolites in the plant, and nanoparticle synthesis, and will be a source for further research in the field of plant-biosynthesis nanotechnology. Additionally, it is of practical importance in optimizing the growing conditions of plants to improve or control the quality of nanoparticles obtained from plants.

Compliance with ethical standards

Disclosure of conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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