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ORIGINAL ARTICLE

Bactericidal activity of Ag nanoparticles biosynthesized from *Capsicum annuum* pericarps against phytopathogenic *Clavibacter michiganensis*

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Abstract

Metallic nanoparticles of different compositions have already found numerous applications in various branches of industry, agriculture, and medicine. Given the well-known antibacterial activity of Ag, silver nanoparticles (AgNPs) are constantly being investigated for their promising ability to fight antibiotic-resistant pathogens. A promising candidate for AgNPs biosynthesis is chili pepper *Capsicum annuum*, cultivated worldwide and known for accumulating significant amounts of active substances. Phytochemical screening of aqueous extract of *C. annuum* pericarps demonstrated accumulation of 4.38 mg/g DW of total capsaicinoids, 14.56 mg GAE/g DW of total phenolic compounds, 1.67 mg QE/g DW of total flavonoids, and 1.03 mg CAE/g DW of total phenolic acids. All determined aromatic compounds carry various active functional groups, which effectively participate in the biosynthesis of AgNPs and are characterized by high antioxidant potential. Therefore, the present research focused on the facile, quick, and effective procedure for the biosynthesis of AgNPs, which were analyzed for their morphology such as shape and size through UV–visible, Fourier-transform infrared spectroscopy (FTIR) assays, and scanning electron microscopy. We found that the AgNPs biosynthesis resulted in changes in FTIR spectra, depicting the rearrangement of numerous functional groups, while the nanoparticles themselves were shown to be stable, spherical, 10–17 nm in size. Also we investigated the antibacterial properties of biosynthesized AgNPs, obtained with *C. annuum* fruit extracts, against a common phytopathogen *Clavibacter michiganensis* subsp. *michiganensis*. As was shown by zone inhibition assay, AgNPs showed dose-dependent 5.13–6.44 cm antibacterial activity, greatly exceeding the 4.98 cm inhibition area, produced by the precursor salt, $AgNO_3$.

Keywords AgNPs · Biosynthesis · *Capsicum annuum* · Antibacterial activity · *Clavibacter michiganensis* subsp. *michiganensis*

Abbreviations

AgNPs	Silver nanoparticles
ANOVA	Analysis of variance
Cmm	*Clavibacter michiganensis* Subsp. *michiganensis*
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
FT-IR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalents
SD	Standard deviation
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
TCC	Total capsaicinoids content
TPC	Total phenolic content
TFC	Total flavonoids content
TPAC	Total phenolic acids content
UV-vis	Ultraviolet-visible spectroscopy

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Introduction

The biosynthesis of silver nanoparticles (AgNPs) with plant extracts is an important subtype of green chemistry approaches. Plant tissues containing specific bioactive compounds are able to reduce and stabilize metallic salts used as precursors in AgNPs synthesis. Such reductive properties against silver ions are known for various molecules that can be identified in the chemical composition of a plant, including ketones, amino acids, aldehydes and vitamins. The low production costs and the profound effectiveness are advantages that can overcome the traditional chemical route of nanoparticle synthesis (Nicolae-Maranciuc et al. 2022). The bioreducers found in plants also include enzymes, proteins, polysaccharides, organic acids and a vast diversity of secondary metabolites such as phenolic compounds and capsaicinoids that are potentially able to reduce metal ions (Smirnov et al. 2021).

Throughout the last decade, AgNPs, biosynthesized from different parts of *Capsicum* species, have received much consideration for various applications due to their substantial ability to disperse in aqueous systems combined with biological compatibility, antibacterial activities, high adsorption and catalytic activity, as well as the ease of their application in chemo- and biosensing fields (Shankar et al. 2017; Kumar et al. 2021; Dzhagan et al. 2022). Extracts enriched with bioreductants, obtained from different species of the genus *Capsicum* (*C. annuum*, *C. frutescens*, *C. baccatum*, and *C. chinense*) were shown to be a good choice for the biosynthesis of metallic nanoparticles. However, few studies have used extracts from plant tissues other than the fruits, such as leaves (Velgosova and Veselovský 2019; Lomelí-Rosales et al. 2022).

Genus *Capsicum* is a member of Solanaceae family and is believed to originate from Central and South America. It is the most commonly cultivated dicotyledonous plant – an indispensable spice used as a basic ingredient in a great variety of cuisines all over the world (Samrot et al. 2018; Rajam et al. 2021). Chilli pepper is a crop of great economic importance, as this vegetable is widely used in traditional cuisine providing flavour, aroma, and colour to various national dishes, and in addition, it is used in food cosmetics and pharmacy (Caicedo-Lopez et al. 2022). Also it has potential applications in herbal and traditional medicine, because it contains compounds such as flavonoids, ascorbic acid, tocopherol, lycopene, minerals and especially capsaicinoids with their antimicrobial, antiseptic, anticancer, counterirritant, appetite stimulator, antioxidant and immunomodulatory activities (Batiha et al. 2020).

The average percentage of food losses may reach 40% worldwide between postharvest and distribution in the fruit and vegetable supply chain including Solanaceae food crops such as tomatoes, potatoes, chilli peppers and eggplants. This food is substantially perishable and often has a fragile physical constitution, leading to a relatively short shelf life (Costa et al. 2021). One of the main infections affecting Solanaceae food crops is the bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) (Solano-Alvarez et al. 2022). The European and Mediterranean Plant Protection Organization classify four subspecies of Cmm as hazardous quarantine microorganisms because of the serious economic danger that they instigate. Solanaceae food crops infected with Cmm strains show diverse symptoms that depend on the host plant cultivar receptivity and virulence of microorganism pathovar, along with specific environmental conditions. Fruits and seeds that are externally contaminated with Cmm strains, contaminated soil, and infected plant debris can serve as an initial source of inoculum for systemic infections (Valencia-Hernandez et al. 2022).

Chemically and physically synthesized AgNPs release silver ions leading to the formation of reactive oxygen species. These radicals in interaction with plants have shown adverse effects, not only by penetrating into the root or shoot, but also by causing physical damage to the surface of plant tissues. Very little attention has been paid concerning the prevalence of bacterial canker and no research has been done on measures for controlling it. The biosynthesis of AgNPs by *C. annuum* pericarps aqueous extract with high bactericidal effects and its ability to induce potential resistance in plants highlights its importance (Noshad et al. 2020; Méndez-Andrade et al. 2022).

In this study, AgNPs were biosynthesized using aqueous extracts of *Capsicum annuum* L. pericarps, which contained aromatic bioactive secondary metabolites with antioxidant activity, and the bactericidal activity of obtained AgNPs was evaluated against phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis* as hazardous and quarantine bacteria.

Materials and methods

Collection and preparation of plant extract

The plant material – pericarps of *Capsicum annuum* L. (cv. 'Teja') were purchased from the popular market near ESC "Institute of Biology and Medicine" in Kyiv, Ukraine. Fresh pericarps of *C. annuum* were washed several times in deionized water for removal of soil and any organic impurities and then air dried at 60 ºC to eliminate the residual moisture. Cleaned and dried pericarps of *C. annuum* were cut into small pieces and powdered into finely dispersed flour. Two grams of plant samples were put in a flask with a flat bottom with 100 ml deionized water and boiled for 20 min at 100 °C. The obtained extracts of pericarps were cooled at room temperature and filtered with Whatman No.1 filter paper.

Phytochemical screening of potential bioreductants

All spectrophotometric assays were performed using UV–vis spectrophotometer UV-1800 "Shimadzu" (Japan). Total capsaicinoids content (TCC) analysis was estimated with Gibbs reagent (2,6-dichloroquinone-4-chloroimide) by the technique described by Ryu et al. 2017 with slight modifications. Total capsaicinojids contents were calculated with the molar extinction coefficient of 2,6-dichlorophenol indophenol according to Armstrong 1964 and expressed in mg per g of dry weight (DW). The total phenolic content (TPC) was determined according to the method with Folin-Ciocalteu reagent, which is based on the quantification of the total concentration of hydroxyl groups that are present in the extract (Boudghane et al. 2022). The absorbance at 765 nm was measured and results were expressed in mg of gallic acid equivalents (GAE) per g of DW. The total flavonoid content (TFC) assay was performed according to the protocol described by Neupane and Lamichlane (Neupane and Lamichhane 2020) with minor modifications. The absorbance at 506 nm was measured and results were expressed as mg of quercetin equivalents (QE) per g of DW. The total phenolic acid content (TPAC) was determined according to the method with Arnova reagent (Vergun et al. 2021). The absorbance at 490 nm was measured and caffeic acid was used as a standard, the results were expressed in mg of caffeic acid equivalents (CAE) per g of DW.

Evaluation of antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a relatively stable radical. Radical scavenging activity determination was used to quantify the ability of bioreductants in plant extracts to put out the DPPH radical within 15 min. Changes in the reaction mixture colouration were measured at 517 nm wavelength (UV-1800 "Shimadzu" (Japan) UV–vis spectrophotometer), which corresponds to the reduction of DPPH$^\bullet$ to a non-radical form. The decrease in absorption correlated with the percent inhibition in samples. The percentage of inhibition was calculated against blank (Nakagawa et al. 2021) as follows:

$$\%\ (AA) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\%,\ where\ (A_0 = control, A_1 = sample) \tag{1}$$

AgNPs biosynthesis

Ag nanoparticles were synthesized using cooled and filtered extracts of *C. annuum* pericarp tissues by the addition of 0.001 M silver nitrate ($AgNO_3$). For the reduction of silver ions and formation of AgNPs, 10 mL of extract was mixed with 40 mL of $AgNO_3$ solution. The resulting solution was incubated under a light-emitting diode lamp (Secret Jardin, 42 W, 6500 K) for 2 h at room temperature for the reduction of silver salt.

Characterization of biosynthesized AgNPs

Ultraviolet spectrophotometric analysis of biosynthesized AgNPs were recorded with UV-1800 "Shimadzu" (Japan) UV–vis spectrophotometer at range of 200–700 nm with 0.5 nm resolution after 2 h of synthesis procedure. Fourier transform infrared spectroscopy (FT-IR, Bruker model VERTEX 80v) was performed to analyse the functional groups of the plant extract and biosynthesized AgNPs in the range of 500 cm^{-1} to 4000 cm^{-1}. The size distribution and morphology was estimated after desiccation of the purified AgNP solutions at 60 ºC by scanning electron microscopy (SEM, Tescan Mira 3 MLU).

Antibacterial activity evaluation

The antibacterial activity of test samples against phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) was evaluated with agar diffusion test method. The culture of the test bacteria was grown in nutrient broth (Himedia) and adjusted to 2.0 McFarland turbidity standards. 1 mL of 72-h Cmm culture suspension was inoculated on the surface of solidified Mueller-Hilton agar in Petri dishes. Then, one well per one dish was made in agar with cylindrical metal tube and 100 µL aliquots of test samples were added to the wells. Commercially available single antibiotic disc impregnated with gentamicin GEN^{30} (Himedia) were aseptically placed on the Mueller–Hinton agar dishes as a positive control. As a negative control, we used the extract of *C. annuum* pericarps. Petri dishes were incubated at 20° C for 6 days and the diameters of zones of inhibition for the six separate determinations were recorded.

Statistical analysis

Each experiment was performed at least in triplicate. The results were expressed as mean ± standard deviation (SD). The analysis of variance (ANOVA) followed by Tukey's

Table 1 Total capsaicinoids, phenolics, flavonoids, phenolic acids, and antioxidant activity of aqueous extract of *Capsicum annuum* pericarps

Aqueous extract	Capsaicinoids[a]	TPC[b]	TFC[c]	TPAC[d]	DPPH-antiradical activity (%)
C. annuum	4.38 ± 0.32	14.56 ± 1.08	1.67 ± 0.14	1.03 ± 0.19	76

Values are means of three replicates ± SD

[a] Total capsaicinoids (mg g^{-1} DW)

[b] Gallic acid equivalent (mg GAE g^{-1} DW)

[c] Quercetin equivalent (mg QE g^{-1} DW)

[d] Caffeic acid equivalent (mg CAE g^{-1} DW)

multiple range test were used for significance tests. Means and standard deviations were calculated using Microsoft Office Excel (Microsoft Office 2010). A value of $P < 0.05$ was considered significant.

Results and discussion

Capsicum annuum is recognized worldwide as irreplaceable crop, valued for its nutritional potential, antioxidant compounds, flavour, pungency, brilliant colours, and texture (Caicedo-Lopez et al. 2022). Almost all parts of the *C. annuum* are considered to be rich in aromatic compounds including polyphenols, flavonoids and capsaicinoids. One of the significant biological properties of bioactive compounds is their ability to act as antioxidants to reduce reactive oxygen species (Akhtar et al. 2021). Determination of total capsaicinoids (TCC), total phenolic compounds (TPC), total flavonoids (TFC) and total phenolic acids (TPAC) contents in extracts from pericarps of *C. annuum* showed the presence all of these compounds in experimental extract (Table 1). As shown in Table 1, experimental aqueous extract demonstrated 4.38 mg/g DW of total capsaicinoids, 14.56 mg GAE/g DW of total phenolic compounds, 1.67 mg QE/g DW of total flavonoids, and 1.03 mg CAE/g DW of total phenolic acids. All determined aromatic compounds carry many active functional groups which effectively participate in biosynthesis of AgNPs and act as probable bioreducing, capping as well as a stabilizing agents and are characterized by high antioxidant potential (Khatoon et al. 2022).

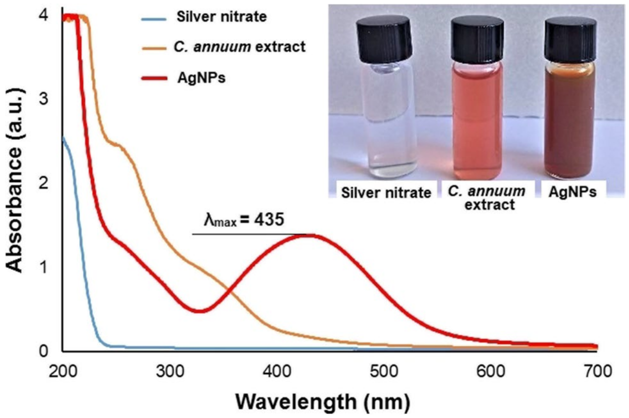

Fig. 1 UV–vis absorption spectra of silver nitrate solution, aqueous extract of *Capsicum annuum* pericarps, and resulting AgNPs solution. The inset shows the changes of colour as the result AgNPs green synthesis – the visible manifestation of the SPR effect

The total antioxidant activity of aqueous extracts of *C. annuum* pericarps was investigated with stable DPPH radical. DPPH$^{\bullet}$ is used in a basic in vitro screening method for evaluating the radical scavenging activity of natural or artificial compounds, different plant extracts because this technique is simple and relatively fast (La et al. 2021). The extract from *C. annuum* pericarps showed the high level of DPPH inhibition at 76% (Table 1).

All identified phytochemicals are potent bioreductive agents. The prominent total antioxidant activity of *C. annuum* pericarps aqueous extracts could be attributed to the substantial amount of TPC. These bioactive molecules present in extracts bear the responsibility for the high DPPH radical scavenging activity and act as reducing agents that in turn play a central role in the process of green synthesis of AgNPs. In addition, there are several studies on the action of capsaicin on AgNPs by the direct reduction of silver nitrate in the aqueous phase, without the use of any other reducing agents (Amruthraj et al. 2015; Dong et al. 2021). Antioxidant activity of extract can affect the experimental optimization of operating parameters needed for the biosynthesis of AgNPs from pericarps of *C. annuum*.

The formation of AgNPs as a result of green synthesis with *C. annuum* pericarp extracts was detected with UV–vis spectrometry. It has been previously shown that solution of metallic nanoparticles shows an increased absorbance at a specific wavelength, which depends on shape, size and composition of nanostructures (Buda et al. 2017; Fahmy et al. 2019). Such optical activity is a result of surface plasmon resonance (SPR) – a characteristic feature of nanoparticles that arises from the interaction of external energy of light with the densely-distributed energy fields on the surface of nanounits (Lee and Jun 2019). In our study, the formation of AgNPs in

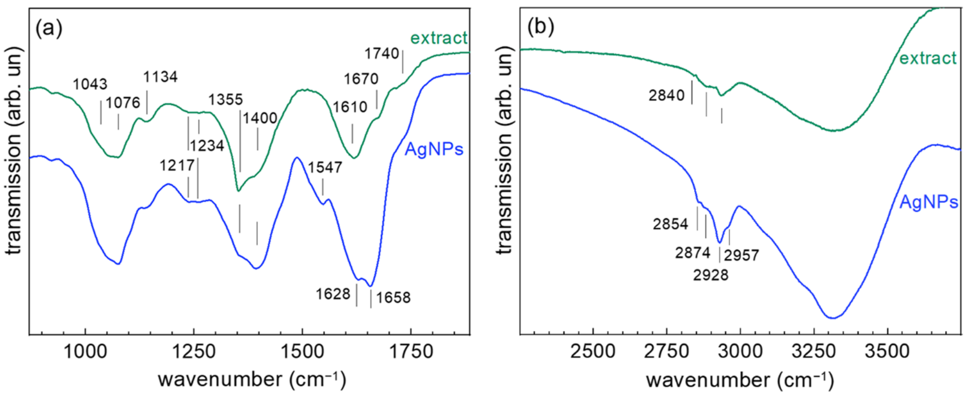

Fig. 2 Infrared transmission spectra of phytosynthesized AgNPs in the range of 700–1900 cm^{-1} (**a**) and of 2400–3600 cm^{-1} (**b**). The spectra shifted vertically for convenience. To avoid overloading the figure, the wavenumbers of absorption peaks are marked only in those spectra where they occur at the largest intensity or are most distinct

experimental solutions was accompanied by the increased absorbance in visible spectrum, with a peak at 435 nm (Fig. 1). After the completion of green synthesis, the width and height of this peak remained constant, proving the stability of obtained AgNP solution. The reduction of silver ions and accumulation of AgNPs could also be easily detected with a naked eye due to increased turbidity of reaction medium, combined with the change of its colour to brown (Fig. 1, inset). These effects are also the result of SPR propagation combined with increased light scattering, which is typical for any nanocolloidal solution (Balachandar et al. 2022; Nayak et al. 2022).

From the FTIR data presented in Fig. 2, we can assume which functional groups or molecular species may be involved in the NP stabilization. The vibration bands that are similar in position and intensity in the pure extract and in AgNP sample are those at 1043, 1076, 1134, 1217, 1234, 1740 cm^{-1} (Fig. 2a), as well as all the peaks in the range 2800–3000 cm^{-1} (Fig. 2b).

The intensity ratio of the bands at 1355 and 1400 cm^{-1} was reversed in the AgNP sample. The same observation is for the pair of bands at 1610 and 1670 cm^{-1}, which in addition change their positions to 1628 and 1658 cm^{-1} in the AgNP sample. Most likely, the functional groups related with these vibrations are involved in the stabilization of the AgNPs. According to the literature, the 1355 cm^{-1} band can be due to N = O of the aliphatic nitro group, reported in Ulaeto et al. 2020 at 1363 cm^{-1} and/or C–H bending in cellulose and hemicellulose, reported around 1365–1375 cm^{-1} (Türker-Kaya and Huck 2017). The 1400 cm^{-1} band can be attributed to C – H bend of alkanes (CH_3), observed at 1395 cm^{-1} in Ulaeto et al. 2020 and/or O–H bending in polysaccharide cell wall, alcohol, and carboxylic acid, observed at 1420–1430 cm^{-1} in Türker-Kaya and Huck 2017.

The candidates for the 1610 (1628) cm^{-1} are C = C of aromatic group, N – H bending in amides according to Ulaeto et al. 2020, C – C skeletal vibrations/N = H deformations, C = O aromatic stretch: lignin, alkaloid (Huang et al. 2008). The 1670 (1658) cm^{-1} band is most likely

Morphological features of obtained AgNPs were described with scanning electron microscopy. Analysis of obtained SEM images (Fig. 3) showed that nanoparticles were uniform and spherical in shape, with an average size of 10–17 nm. Notably, we detected low number of AgNP aggregates, while the main part of the solution was represented with finely-dispersed nanostructures.

It is known that stability of nanoparticles greatly depends on the exact composition of the solution as well as concentration of particular molecules. Specifically, the presence of capping agents in the reaction medium during nanoparticle formation enables effective creation of the protective layer on AgNPs, promoting their repulsion and,

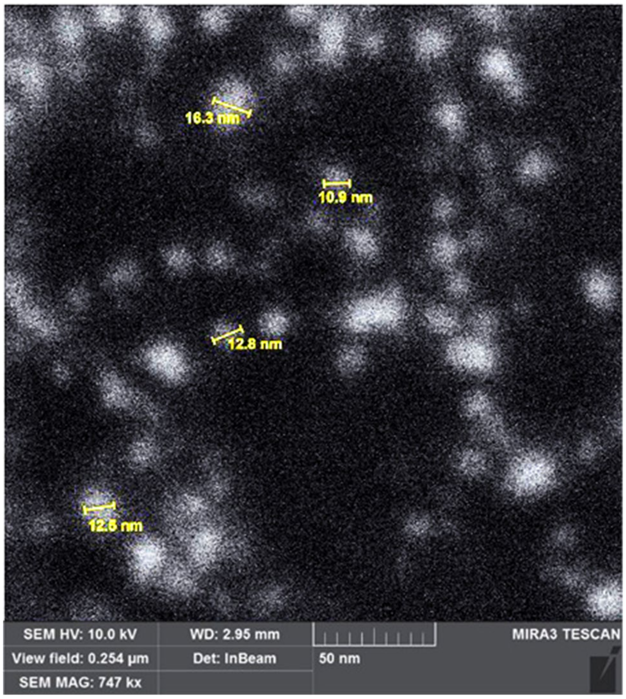

Fig. 3 Micrograph image of scanning electron microscopy of AgNPs obtained by biosynthesis using aqueous extracts of *Capsicum annuum* pericarps

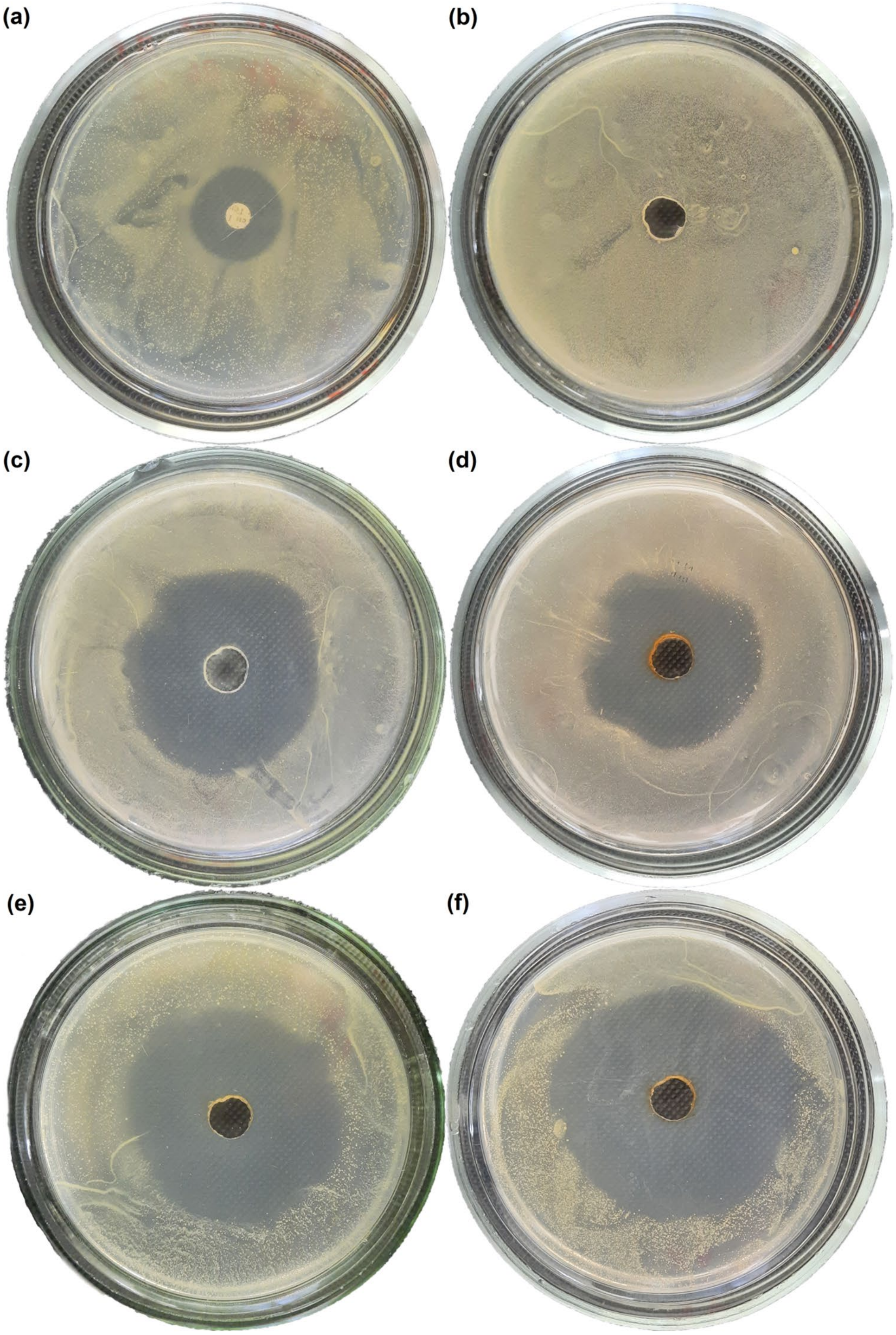

Fig. 4 Antimicrobial susceptibility agar diffusion method against *Clavibacter michiganensis* subsp. *michiganensis*: zone of inhibition of gentamicin (**a**), extract of dry pericarps *Capsicum annuum* (**b**), 0.001 M silver nitrate (**c**), AgNPs in 20 mg/L (**d**), 40 mg/L (**e**), 80 mg/L (**f**) obtained by biosynthesis using aqueous extracts of *Capsicum annuum* pericarps

related with amide I (C = O stretch) in protein, pectin, water associated cellulose or lignin, alkaloids (Türker-Kaya and Huck 2017).

The band at 1547 cm^{-1} is not registered for bare extract, only for the NP sample. The vibrations around this frequency were attributed in the related literature (on phytoextracts) to N – H bending in amides & the interaction between N – H bending and C – N stretching of C – N–H group and amide II (C = N and N–H stretch) in protein (Türker-Kaya and Huck 2017).

Table 2 Zone of inhibition diameter (cm) of experimental solutions against *Clavibacter michiganensis* subsp. *michiganensis.* The mean and standard deviation (SD) reported for each type of experimental solution were based on six biological replicates: means followed by the same letters were not significantly different at $P < 0.05$ according to the Tukey's multiple range test

Experimental variants	Gentamicin	Extract	$AgNO_3$	20 mg/L	40 mg/L	80 mg/L
Zone of inhibition (cm)	1.92 ± 0.12^a	N/A*	4.98 ± 0.16^b	5.13 ± 0.21^b	5.86 ± 0.32^c	6.44 ± 0.18^d

*N/A – not available

thus, decreasing the aggregation tendency (Ali et al. 2021; Bélteky et al. 2019). We therefore might postulate that bioactive compounds in *C. annuum* extract may serve as effective stabilizing agents for AgNPs. Silver nanoparticles of similar size, shape and stability were also obtained in other plant-based green synthesis approaches. For instance, Salayová et al. 2021 received 15–75 nm AgNPs with extracts from five different plant species, while Ahn et al. 2019 reported synthesis of 8–35 nm particles with 30 different extracts. All authors underline that properties of obtained AgNPs are specific for the plant species and therefore the selection of suitable source of bioreducing compounds may affect the possible range of applications of green-synthesized AgNPs.

The antimicrobial activity of the biosynthesized AgNPs in different concentrations (20 mg/L, 40 mg/L, and 80 mg/L) was assessed against phytopathogenic bacteria *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). The results indicated that test samples exhibited varying antimicrobial activities against the tested phytopathogen. Antibacterial activity was estimated by the size of the zone of bacterial culture growth inhibition in comparison with the gentamicin, 0.001 M silver nitrate solution, and the primary extract of *C. annuum* pericarps (Fig. 4).

Gentamicin, which was used as positive control, is an aminoglycoside antibiotic used in the treatment of several bacterial infections. The highest antimicrobial activity was recorded for the AgNPs at a concentration of 80 mg/L (6.44 cm) followed by those for the AgNPs at a concentration of 40 mg/L (5.86 cm). The effect of experimental samples of AgNPs at a concentration of 20 mg/L and 0.001 M silver nitrate solution as precursor salt were not statistically significantly different (5.13 cm and 4.98 cm respectively). In contrast, the lowest antimicrobial action was recorded for gentamicin influence (1.92 cm), at the same time primary extract of *C. annuum* pericarps did not affect Cmm bacterial culture at all (Table 2).

The activity of biosynthesized AgNPs against Cmm bacterial culture was also evaluated by Noshad et al. 2019, where filamentous fungi *A. fumigatus* and *T. harzianum* were used as bioreducers. However, our results indicate the prevalent anti-Cmm activity of *C. annuum*-derived AgNPs, proposing its potential wide application for food processing and agriculture.

Conclusions

The present study confirms the possibility of AgNPs biosynthesis by aqueous extracts of *Capsicum annum* pericarps. The phytoscreening of plant-derived extract showed accumulation of bioreducing compounds, whose activity was confirmed by the DPPH quenching assay. The obtained AgNP solutions showed remarkable stability combined with structural uniformity of nanostructures, as verified by UV–vis, SEM and FT-IR analyses. Plant-derived AgNPs showed excellent antibacterial activity against phytopathogen *Clavibacter michiganensis* subsp. *michiganensis*, compared to the traditional antibiotic gentamycin. Therefore, *C. annuum* may be used as an effective and eco-friendly source of bioactive AgNPs for their application in agriculture and food technology.

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Author contributions Oleksandr Smirnov and Vitalii Kalynovskyi project conceptualization and edited the manuscript, Pavlina Zelena and Yuliia Yumyna designed the experiments with microorganisms, Volodymyr Dzhagan performed the FTIR analysis, Mariia Kovalenko wrote the first draft of the manuscript, Yevheniia Konotop and Nataliya Taran analyzed the manuscript contents and made the manuscript corrections. All authors have read and agreed to the published version of the manuscript.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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