

THE STUDY OF ANTIMICROBIAL ACTIVITY OF AgNPs MADE BY USING GREEN SYNTHESIS AGAINST SPECIFIC MICROORGANISMS

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Abstract

This work is focused on studying the antimicrobial effect of silver nanoparticles made by green synthesis. For the green synthesis, extracts from *Salvia officinalis* were used. Four different temperatures (20, 40, 60, 80 °C) were used to prepare the extracts from the plant material. The antimicrobial capability of the AgNPs was assessed using different yeasts (*Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*), gram-positive (*Lactobacillus plantarum*, *Listeria innocua*, *Staphylococcus Aureus*) and gram-negative (*Escherichia coli*, *Acetobacter aceti*) bacterial strains. The range of AgNPs concentrations 0-2,500 µg/mL were tested. The antimicrobial effect was studied using two methods: 1) the viable cell count method and 2) the inhibition zone method. The method of direct counting showed a small inhibitory effect (inhibition at concentrations 1,250 and 2,500 µg/ml) only for *E. coli*, *Z. bailii* and *S. cerevisiae*. The inhibition zone results displayed better antibacterial activity of the silver nanoparticles (inhibition at all concentrations - 50, 100, 150 and 200 µg/mL) prepared by using the extract obtained at 20 °C against *E. coli* and *S. aureus*.

Keywords: Green synthesis, silver nanoparticles, antimicrobial activity

1. INTRODUCTION

The process of green synthesis in nanoparticle production has become a more and more popular alternative to chemical synthesis in recent years. It is a simple, inexpensive and environmentally friendly method for producing nanoparticles. Biosynthesised nanoparticles show high biocompatibility and antimicrobial, antioxidant, antidiabetic, anticancer and other effects, and thus have the potential to be widely used in many fields [1,2].

Silver nanoparticles produced by green synthesis are abundantly studied for their antimicrobial properties [3,4]. This is primarily based on the biological matrix of the sample. Parameters in the production of nanoparticles such as temperature, pH, extraction time and individual production processes play an important role [5,6].

Salvia officinalis, used in our work as a reducing agent, contains a significant amount of antioxidant components. It contains essential oils, tannins, phenolic substances and flavonoids [7,8]. The production of essential oils, which are contained in the leaves, is significant [9].

This work aimed to determine the inhibitory effect of AgNPs on selected bacteria and yeasts. In the second part, the aim was to test the effect of AgNPs prepared at various temperatures against *S. aureus* and *E. coli*.

2. MATERIALS AND METHODS

2.1. Preparation of AgNPs by green synthesis

Preparation of AgNPs by green synthesis is shown in **Figure 1 A**. Plant materials (*Salvia officinalis*) were dried at 60 °C for 48 h and homogenised by grinding to dust. For the preparation of the plant extract, the mixture was stirred in water (20, 40, 60, 80 °C) for 60 minutes in a ratio of 1:10 followed by centrifugation (15 min; 4,000 g). The extract was mixed with 0.1 M AgNO₃ (1:1; 24 h), and the prepared particles were purified with methanol (1:1; 1 h). After precipitation, the methanol was removed, and the AgNPs were dried at 60 °C for 24 h in VWR (Radnor, Pennsylvania, USA) dryer (model VDL23). The purified AgNPs were dispersed in a medium (purified water 18 MΩ or a mixture of water/acetone) by using ultrasound (40 minutes until a homogenous mixture was obtained) to obtain a stock solution of nanoparticles of 10 mg/mL.

AgNPs were characterised by UV-VIS spectra, XRD, spectra, dynamic light (DLS) and transmission electron microscopy (**Figures 1 B, C, D, E**).

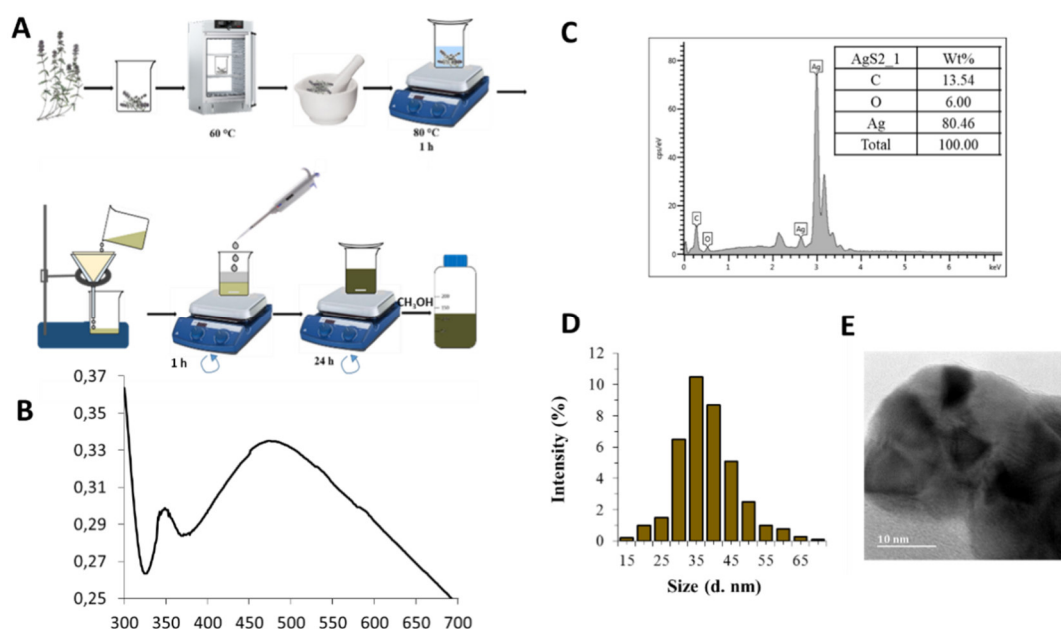


Figure 1 A) Simplified scheme of green synthesis of AgNPs. **B)** Typical absorbance spectrum with the maximum signal in the area of 450 nm. **C)** XRD spectrum showing the presence of carbon and oxygen on the surface of AgNPs. **D)** The average hydrodynamic size of AgNPs measured by zeta sizer. **E)** TEM picture of AgNPs.

2.2. Culture conditions and strains

The reference strains for the first experiment (*S. cerevisiae*, *Z. bailii*, *L. plantarum*, *L. innocua*, *E. coli* and *A. acetii*) were obtained from the Colección Española de Cultivos Tipo (CECT; Valencia, Spain). The reference strains for the second experiment (*E. coli* and *S. aureus*) were obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic).

The cultures of microorganisms were stored at 4 °C in plate count agar before use. The cells from a colony of microorganisms grown on agar PCA (for *E. coli* and *L. innocua*) MRS (for *L. plantarum* and *A. acetii*) or YPDA (for *S. cerevisiae* and *Z. bailii*) were transferred to 10 mL of broth and were incubated at 30 °C (for *S. cerevisiae*, *Z. bailii*, *L. plantarum* and *A. acetii*) or 37 °C (for *E. coli*, *L. innocua* and *S. aureus*) for 24 h to obtain an inoculum with a density of approximately 1 x 10⁷ cells/mL of broth.

2.3. Antimicrobial susceptibility assays

For the viable cell count method: The antimicrobial activity of the AgNPs prepared by green synthesis was determined by the microdilution method [10] with some modifications, within a range of concentration of 0, 156.25, 312.5, 625, 1,250 and 2,500 µg/mL. For this experiment, AgNPs made at 20 °C were used in all cases. Different volumes of the stock solution (10 mg/mL) and broth media were added to 96-well microtiter plates to obtain tested concentrations with a final volume of 200 µL. Then a 1-10 µL of the microbial inoculums were inoculated in microtiter plate wells to obtain a final concentration of 10⁴ cells/mL. The plates were incubated at 30 °C (for *S. cerevisiae*, *Z. bailii*, *L. plantarum* and *A. aceti*) or 37 °C (for *E. coli* and *L. innocua*) for 24 h. Then 100 µL of samples were collected and spread on Petri dishes with PCA, MRS or YPDA. The plates were incubated for 24 h at 30 °C (for *S. cerevisiae*, *Z. bailii*, *L. plantarum* and *A. aceti*) or 37 °C (for *E. coli* and *L. innocua*) and after incubation, viable cell numbers were enumerated, and the colony-forming units per millilitre (CFU/mL) were determined. These values were logarithmically transformed and expressed as log CFU/mL. The treatments were performed with at least six replicates for each condition. Positive and negative controls were included in all assays.

For the inhibition zone method [11]: Petri dishes with agar medium were covered by 50 µL of bacteria suspension (OD₆₂₀ = 0.01). AgNPs (c = 10 mg/mL) dispersion was pipetted into a Petri dish in amounts of 5, 10, 15 and 20 µL. This resulted in variants with concentrations of 50, 100, 150 and nd of 200 µg/mL. The bacteria were cultivated for 12 h at 37 °C. After 12 h of incubation, photographs were taken, and the total area of the inhibition zone in cm² was calculated in the LIS programme (Mediapro, s.r.o., Brno, Czech Republic). When the inhibition zone began to overgrow with bacterial colonies, a constant value of 0.01 cm² was selected.

3. RESULTS

3.1. Viable cell count method results

This method allows for identifying the number of viable cells in a given culture. Specifically, it involves counting colonies that are visible to the naked eye. **Table 1** shows the microbial counts of the studied bacteria and yeast in the presence of different concentrations of the AgNPs.

Table 1 Microbial counts (log CFU/mL) of the different microorganisms after incubation with the AgNPs

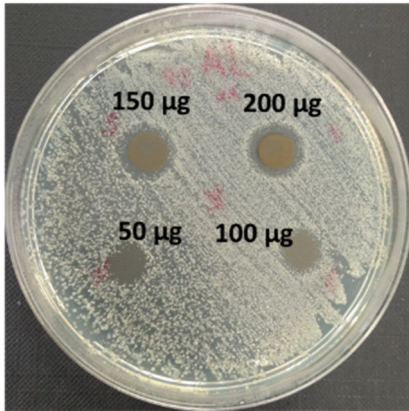
Microorganisms / Concentration	2,500 µg/ml	1,250 µg/ml	625 µg/ml	312.5 µg/ml	156.25 µg/ml	0 µg/ml
<i>E. coli</i>	0.86	1.18	>4	>4	>4	>4
<i>Z. bailii</i>	3.30	3.93	>4	>4	>4	>4
<i>S. cerevisiae</i>	3.91	>4	>4	>4	>4	>4
<i>L. plantarum</i>	>4	>4	>4	>4	>4	>4
<i>L. innocua</i>	>4	>4	>4	>4	>4	>4
<i>A. aceti</i>	>4	>4	>4	>4	>4	>4

The results of the study show us that the silver nanoparticles have a relatively small antimicrobial effect. This occurred only in the bacterial culture of *E. coli* and yeast cultures of *Z. bailii* and *S. cerevisiae*. The effect was observed only at concentrations of 1,250 and 2,500 µg/mL, and in *E. coli* only at concentrations of 2,500 µg/mL.

3.2. Zone inhibition results

In the next part of the experimental work, the effect of the AgNPs on *E. coli* and *S. aureus* was studied using the zone inhibition method. In this method, the size of the area around each disc indicates the antimicrobial effect of the individual samples of AgNPs. For this, nanoparticles produced at 20, 40, 60 and 80 °C were used and pipetted into Petri dishes at concentrations of 50, 100, 150 and 200 µg of AgNPs/mL (**Figures 2A, 2B**).

A) Inhibition zones of AgNPs (*S. aureus*)



B) Inhibition zones of AgNPs (*E. coli*)

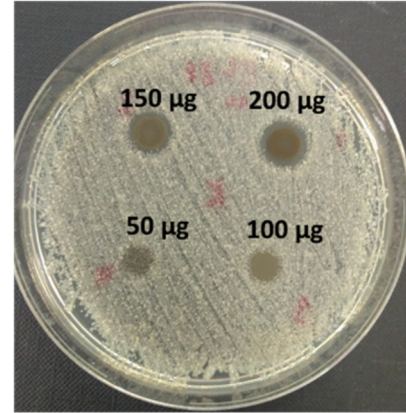


Figure 2 Typical view of inhibition zones of the agar diffusion test

Box plots showing the average value of the region of the inhibition zone were constructed from the obtained inhibition zone results (Figure 3).

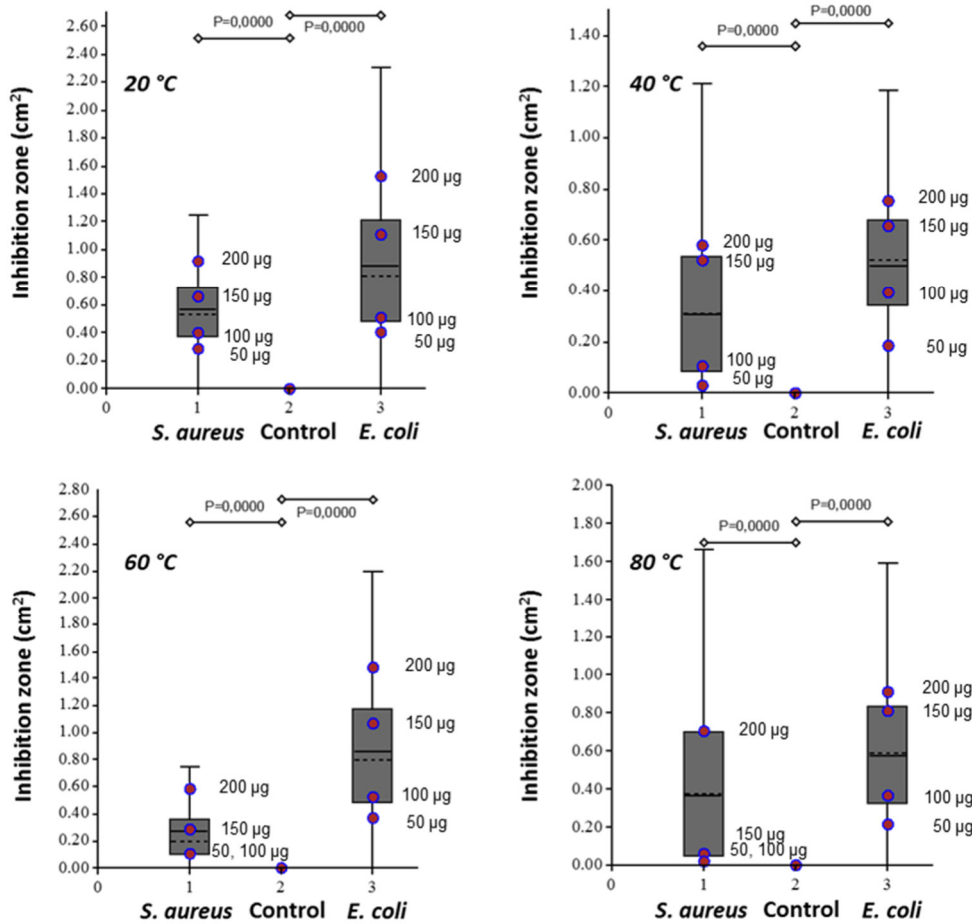


Figure 3 Box plots comparing the area of inhibition zone of AgNPs against *E. coli* and *S. aureus*. Purified sterilised water was used as a control. Four different temperatures (20, 40, 60, 80 °C) were used in the process of preparing the plant extract, and four different AgNPs concentrations were tested (50, 100, 150 and 200 µg/mL)

Box plots comparing the area of the inhibition zones of AgNPs to *S. aureus* and *E. coli*. The purified water was used as a control. Inhibitor zone sizes ranged from 0.01 cm² to 1.5 cm². The best antibacterial effect on *S. aureus* was found with the AgNPs prepared by using the extract that was prepared at 20 °C (value 0.9 cm² at concentration 200 µg/mL). The best effect on *E. coli* also had AgNPs prepared by using the extract at 20 °C (value 1.5 cm² at concentration 200 µg/mL). A better antibacterial effect of AgNPs was obtained against *E. coli*.

4. CONCLUSION

In this experimental work, silver nanoparticles were prepared using green synthesis. In the first part of the experimental work, when the 100 µL nanoparticle solution was spread on Petri dishes and subsequently calculated by CFU, we were able to show a small inhibitory effect only in the bacterial culture of *E. coli* and yeast cultures of *Z. bailii* and *S. cerevisiae*.

In the second part of the experiment, four different amounts (50, 100, 150 and 200 µg of AgNPs/mL) produced at four different temperatures (20, 40, 60 and 80 °C) were pipetted onto the Petri dishes. The detection of differences in the size of the inhibition zones in the preparation at different temperatures was critical. The best antibacterial effect to *E. coli* and also *S. aureus* was found with the AgNPs prepared by using the extract that was prepared at 20 °C. In the future we anticipate the study of antimicrobial activity using different extraction times of the biological matrix and other solvents.

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