PREPARATION OF SILVER NANOPARTICLES BY GREEN SYNTHESIS OF VINE SEEDS – ANTIBACTERIAL EFFECT

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Abstract

Several silver nanoparticles (AgNPs) with biological effect were prepared by green synthesis. AgNPs were prepared from *Vitis vinifera* seeds (Merlos variety) used as wine production waste. Green synthesis brings intensive surface modifications of nanoparticles. The seeds were lyophilized and then homogeneously ground. Purified extracts (0.5, 2.5, 5 and 10 g / 100 mL water) were added to $AgNO_3$ solution(1 M) in a 1:1(v/v) ratio and stirred for 24 h. The formed nanoparticles were precipitated with methanol (1:1) (v/v) and lyophilized. Secondary metabolites were analyzed by various methods. The preparation yield of AgNPs ranged between 3 - 25 %. The total protein values determined by the Lowry method ranged between 1.8–3.6 mg/mL. Antioxidant activity values determined by the CUPRAC method ranged between 76 – 157 µg/mL GAE. Total phenols values determined by Folin-Ciocalteu ranged between 88 – 160 µg/mL GAE, by 4-aminoantipyrine method (TAAP) ranged between 728 - 1,299 µg/mL GAE, by Price Butler method (PBM) ranged between 57 - 108 µg / mL GAE. AgNPs prepared from *Vitis vinifera* seeds (AgNPs-VV) showed also antibacterial activity. Minimal inhibition concentration (MIC) of AgNPs-VV in *S.aureus* was 31 µg/mL and MIC in *E. coli* was 55 µg/mL.

Keywords: Green synthesis, phenolic compounds, analytical methods, antibacterial effects; traditional medicine, ethnobotany

1. INTRODUCTION

Chemotherapeutics based on natural sources are very intensively searched and studied [1,2]. Secondary plant metabolites involve a number of groups of substances [3]. At the beginning of the 21st century there is still a

major concern of infections arising from improper use of antibiotics, disinfectants in hospitals and still not well-understood mechanisms [4]. Preventing these infections is not easy [4]. According to statistics, more people die from bacterial infections than from injuries and cancer [5,6]. The observed rapid rise in bacterial resistance requires the search for new strategies [6]. One of the possible solutions seems to be the use of nanotechnologies [7]. Some types of nanoparticles, including silver nanoparticles (AgNPs), show antimicrobial, antiviral and antifungal effects [8]. In addition, green synthesis of AgNPs uses enzymes and plant extracts [8]. It has lower costs, is environmentally friendly and does not require the use of high pressure and temperature [8]. In addition, important groups are represented by phenolic structures, which have antitumor, antiviral and antibacterial properties [9]. Stilbenes are very important in this area [10]. Stilbenes are part of foods and beverages such as blueberries, peanuts, grapes and red wine [11]. Vine is one of the most important sources of phenolic compounds (including stilbenes). These phenols are present in the skins and seeds [12] and they are shown in **Figure 1**. Resveratrol is the most studied stilbene, which has positive health effects [10]. The wine industry produces a number of wastes (wood, cane, and root) in the production of wine. Modification of the nanoparticle surface with these secondary metabolites can bring a number of completely new properties that can be used in biological applications [13].

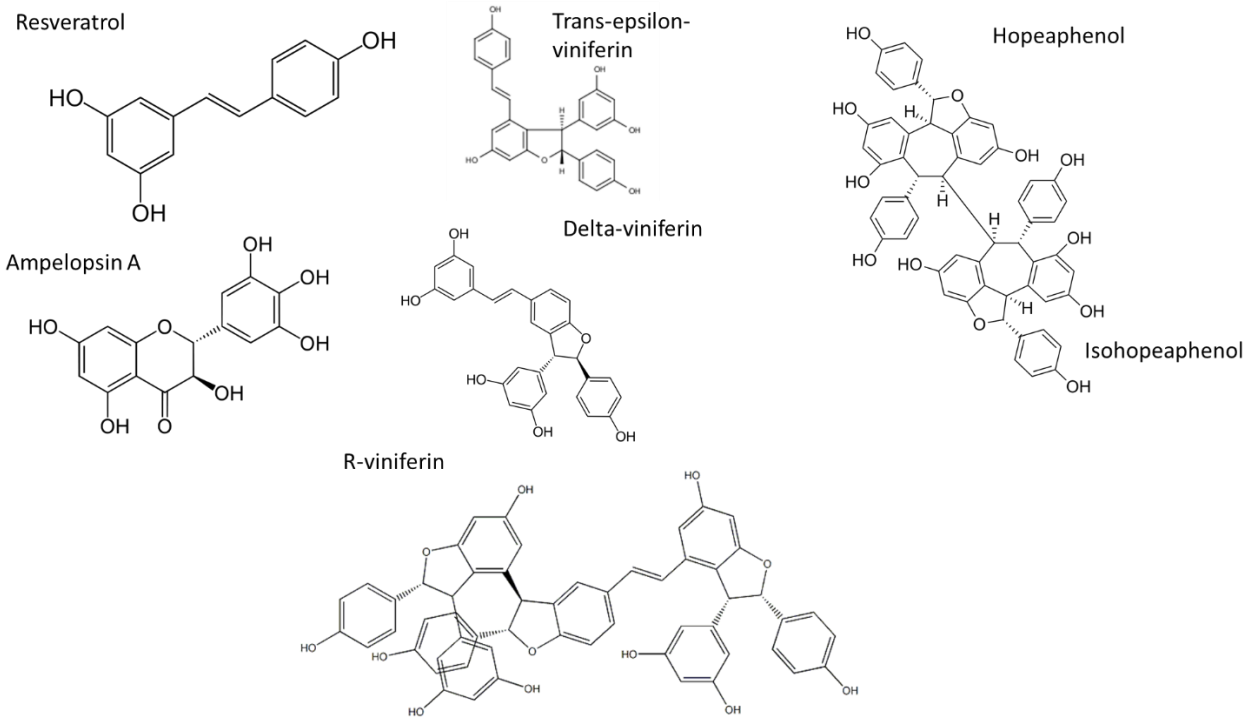

Figure 1 Oligostilbens are a major component of stilbenes extracted from these wastes. An important group of stilbenes in wine are: resveratrol, ampelopsin A, trans-epsilon-viniferin, delta-viniferin, hopeaphenol, isohopeaphenol, and r-viniferin [14,15].

The aim of this work was to design new types of AgNPs from the waste product of *Vitis vinifera* seeds and to test their antibacterial activity.

2. MATERIAL AND METHODS

AgNPs from Vitis vinifera AgNPs-VV. Short description of nanoparticle preparation: Immediately after collection, the plants were washed in distilled water and divided into smaller parts. Seeds of grape Merlot (Lednice, Czechia) were obtained during processing of the grapes into wine. The seeds were washed and lyophilized (48 h, -80 °C, 1 mbar), then homogenized by grinding, using a laboratory grinder (Retsch, GM 200,

Germany) (3 min, 10,000 rpm). The material thus prepared was used to prepare the plant extract. Plant extracts were prepared from various amounts of plant material (0.5; 2.5; 5.0; 10.0 g / 100 mL ultrapure water). The extracts were stirred at 25 °C, on a magnetic stirrer (600 rpm, 60 min). They were then centrifuged (20 min, 14,000 g). Each plant extract was filtered and allowed to cool to room temperature. After filtration, the plant extracts were mixed with 0.1 M $AgNO_3$ in a 1:1(v/v) ratio and allowed to stir with a magnetic stirrer (IKA RH basic, Malaysia) at room temperature (23 °C) for 18 hours. The purified nanoparticles were centrifuged (14,000 g, 30 min) and the pellet was lyophilized (24 h, -77 °C, 0.6 mbar). The yield of the reaction was determined.

Photometric characterization of the extract. VIS absorption spectra were measured in the 400–750 nm range. During the formation of nanoparticles, the colour of the resulting solution gradually changed (increasing the absorbance in the area of 450 nm due to the formation of nanoparticles). (Biochemical analysis of AgNPs surface was performed on BS-300 automatic analyzer (Mindray, China). DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and FRAP (ferric reducing ability of plasma) methods were used for sample analysis [16,17]. The extracts were characterized by spectrophotometric methods for the content of phenolic substances (Folin Ciocalteau, 4-aminoantipyrine method - TAAP, Price Butler method - PMB) and proteins (Lowry method, pyrogallol red).

Antibacterial activity of AgNPs-VV. Escherichia coli a *Staphylococcus aureus* were obtained from the Collection of Microorganisms of Masaryk University Brno (cultivation of stock culture LB medium, 37 °C, 24 h). Infinite F50 (Tecan, Switzerland) was used for measurements. The antibacterial activity of the AgNPs-VV prepared by green synthesis was determined by the microdilution method, with some modifications. Different volumes of the stock solution AgNPs-VV and broth media were added to 96-well microtiter plates to obtain tested concentrations with a final volume of 250 µL. Then a 1-10 µL of the microbial inoculums were inoculated in microtiter plate wells to obtain a final concentration of 10^4 cells/mL. The plates were incubated for 24 h at 30 ºC and after incubation, viable cell numbers were enumerated, and the CFU/mL were determined. The growth curves were determined for 24 h at 30 °C in a sterile microtiter plate (0.3 ml, shaking 5 s, 150 rpm). Measurements were performed on an Infinite 50 (Tecan, Switzerland) at 620 nm. Absorbances were recorded every 15 min.

Mathematical and statistical analysis. Experimental work was performed in at least three independent experiments. Each sample in the experiments was analysed at least five times. The obtained data presented in this paper are the average values. No experimental points were excluded from the proposed experimental study. All the obtained data were stored in the Qinslab database. If possible, data were processed and evaluated mathematically and statistically in the Qinslab database. The results were expressed as mean ± standard deviation (SD). Positive and negative controls were included in all assays. Probit analysis used to determine the minimum inhibitory concentration (MIC).

3. RESULTS AND DISUSSION:

3.1. Preparation of new AgNPs-VV from Vitis vinifera seed (Merlos variety)

In our previous experimental work, approaches for the synthesis and characterization of AgNPs were optimized [18]. The effect of obtaining dry and dispersed nanoparticles on the time of sonication was studied in detail on AgNPs prepared with extracts of tea, wormwood and sage (the effect was studied as an increase in the A450 absorbance signal). Elevated temperature drying (60 °C, 24 h) and lyophilization (-80 °C, 0.1 mbar, 48 h) were used to dry the nanoparticles. After the drying process, the nanoparticles were prepared at a concentration of 10 mg / mL. They were then dispersed in a conventional ultrasonic bath (4 W for a maximum of 300 minutes). For AgNPs prepared using extracts of Vietnamese tea, wormwood, sage after drying and dispersion, the absorbance ranged 0.1 – 1.4; 0.05 – 1.0; 0.05 – 0.6 ($y = -0.0934 + 0.0124\ x + 0.00001\ x^2$, $R^2 = 0.988$; $y = -0.0114 + 0.0030\ x$, $R^2 = 0.976$; $y = 0.0143 + 0.0009\ x + 0{,}00001\ x^2$, $R^2 = 0{,}953$), respectively and after

lyophilization, the absorbance ranged 0.6 -1.60; 0.1 – 1.2; 0.2 – 1.0 ($y = 0.4600 + 0.0077 x + 0.00001x^2$, $R^2 = 0.991$; $y = 0.1683 + 0.0032 x$, $R^2 = 0.976$; $y = 0.1475 + 0.0058 x + 0.00001x^2$, $R^2 = 0.993$), respectively. The results clearly showed that the lyophilization process improves the dispersibility of the nanoparticles and the process further depends on the type of extract used. We applied these conditions for the preparation of new AgNPs-VV. Extracted from lyophilized seeds in amounts (0.5; 2.5; 5.0; 10.0 g / 100 mL) were prepared. The prepared extracts were characterized by visual coloration. The yield increased (from 3 to 25 %) linearly depending on the amount used ($y = 2.339x + 1.771$, $r = 0.999$). According to the optimized procedure, AgNPs-VV were prepared, in which we focused on the introduction of new methods for the evaluation of flavonoids present in grapevine seeds.

3.2. Automated analysis of phenolic compounds in *V. vinifera* extracts

New methods have been introduced for the characterization of plant extracts and silver nanoparticles in terms of quantification of phenolic substances, proteins, antioxidant activity and flavonones. CUPRAC method, which is based on the principle of reduction of Cu^{2+} -neocuproine complex to Cu^{+} -neocuproine due to antioxidants present in the sample (**Figure 2**). The measurement of the resulting complex was at 450 nm. In addition, it was possible to fully automate the method. As a control for the stability of the method, a control chart was created for the gallic acid concentration of 25 µg / mL and 12.5 µg / mL, where the average value of the control chart for 7 days (58804 ± 1964 mAU and 34461 ± 2360 mAU).

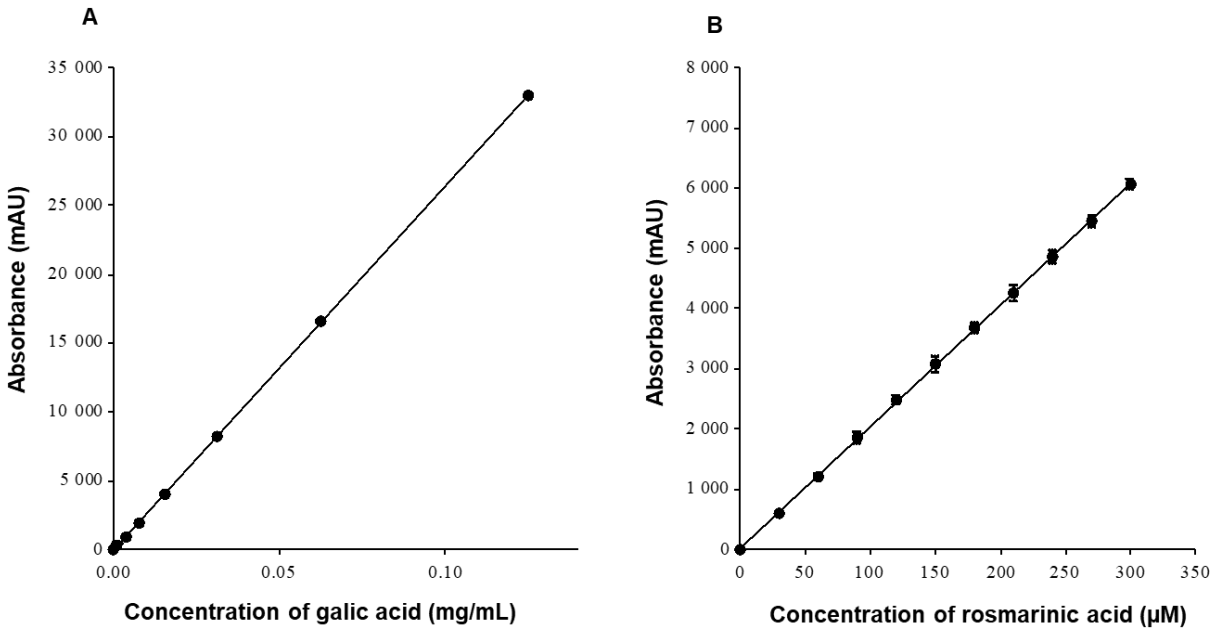

Figure 2 Evaluation of antioxidant activity using the CUPRAC method. The activity is expressed as the equivalent of gallic acid (A). Typical dependence of absorbance on rosmarinic acid concentration (B).

The FLAVANONES method, which is based on the reaction of flavanones of plant extracts with 2,4-DNPH and after subsequent alkalization, results in a quantitative black-blue coloration of the solution. The measurement of the resulting complex was at 505 nm and the method could be fully automated. As a control for the stability of the method, a control chart was created for the concentration of hesperetin 6 µg / mL, where the average value of the control chart for 7 days (4012 ± 323 mAU). The Price Butler method (PBM), which is based on the reaction of a mixture of ferric chloride and potassium ferrocyanide with phenolic substances in a slightly acidic medium. The measurement of the color complex was at 740 nm and the method could be fully automated. As a control for the stability of the method, a control diagram for the concentration of gallic acid 13 µg / mL was created, where the average value of the control diagram for 7 days (58068 ± 3573 mAU). TAAP method, which

is based on the reaction of a mixture of potassium ferricyanide and 4-aminoantipyrine in an alkaline medium. The measurement of the resulting complex was at 450 nm and the method could be fully automated. As a control for the stability of the method, a control diagram for the gallic acid concentration of 53 µg / mL was created, where the average value of the control diagram for 7 days (3947 ± 368 mAU). The methods used showed very good reproducibility and stability during the analysis of the obtained samples. Individual analyzes were performed in the used plant extracts. We found that the content of total phenols determined by the Folin-Ciocalteu method ranged from 0.2 to 1.4 mg / mL GAE, by TAAP method ranged between 0.1-1.2 mg/mL GAE, by Price Butler method (PMB) ranged between 0.1 - 0.8 mg/mL GAE. We paid special attention to the analysis of flavanones. Their content ranged from 0.07 to 0.47 mg / mL hesperetin, 0.06 to 1.17 mg / mL silimarin and by CUPRAC method from 0.13 to 0.95 mg / mL GAE. The amount of total protein ranged from 1 to 6 mg / mL of extract. Analyzes were also performed in AgNPs-VV. Total phenols values determined by Folin-Ciocalteu ranged between 88 – 160 µg/mL GAE, by TAAP method ranged between 728 - 1,299 µg/mL GAE, by PBM method ranged between 57 - 108 µg / mL GAE. The total protein values determined by the Lowry method ranged between 1.8–3.6 mg/mL.

3.3 AgNPs-VV antibacterial activity

Developing bactericidal surfaces using simple chemical methods can be a very promising way to many applications. Antibacterial activity was determined on model organisms (*S. aureus, E. coli*). Growth curves were measured for individual bacterial strains (24 h). We found that AgNPs-VV showed 35-50% inhibitory activity of the control (*S.aureus* or *E.coli* without AgNPs-VV). AgNPs-VV showed higher antibacterial activity against *S. aureus* and lower antibacterial activity against *E. coli*. Based on the calculation of the IC_{50}, the MIC of AgNPs-VV in *S.aureus* was determined to be 31 µg/mL and MIC of AgNPs-VV in *E.coli* was 55 µg/mL.

4. CONCLUSION

There is still very little information on the behavior of nanoparticles in the environment. In addition to chemical synthesis, green synthesis is an alternative that can improve the degradability of nanoparticles from the environment. AgNPs-VV were prepared from several extracts from *V. vinifera* seeds. Total phenols were determined by different methods in both seed extracts and AgNPs-VV. It was shown, that AgNPs-VV contained phenols and had antioxidant activity. AgNPs-VV also showed antibacterial activity against both *S.aureus* and *E.coli*. Our results show that the application of green chemistry processes improves the synthesis of AgNPs and probably improves their potential use in biological applications.

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