Aqueous extract of strawberry (*Fragaria x ananassa* Duch.) leaves as a stabilizing agent in the synthesis of bio-active silver nanoparticles

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Abstract

The aim of the presented work was to investigate the potential of aqueous extract of cultivated strawberry (*Fragaria x ananassa* Duch.) leaves for stabilization of silver nanoparticles (AgNPs-E) synthesized at room (RT) and boiling temperature (BT). The synthesis and stability of AgNPs-E were monitored by UV-Vis spectroscopy confirming high stability of the AgNPs-E in the dark at room temperature. The Fourier-transform infrared spectra suggest that molecules containing oxygen and nitrogen functional groups (NH, (NH)C=O, CNO, C-O-C and OH) participate in the reduction and stabilization of formed nanoparticles. As determined by the DPPH test, AgNPs-E synthesized at RT exerted higher antioxidant activity as compared to AgNPs-E synthesized at BT (EC_{50} values of 0.025 and 0.039 mg cm^{-3}, respectively). Also, the AgNPs-E synthesized at RT exerted higher antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis* and *Bacillus luteus*. Examination of the AgNPs-E on HeLa and MDCK cell lines showed concentration-dependent and cell line specific effects on the cell viability as evaluated by the MTT test. The obtained results indicate that synthesized AgNPs-E can be used as a base material in production of pharmaceutical preparations for potential skin applications.

Keywords: AgNPs; plant extract; green synthesis; antibacterial activity; anticancer activity.

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1. INTRODUCTION

Biosynthesis of nanoparticles is gaining an increasing attention as a multidisciplinary field due to the growing need for development of environmentally friendly technologies [1]. Various types of metal nanoparticles such as copper, gold, palladium, rhodium, platinum, silver, iron *etc.* have been developed for biomedical applications [2]. Silver is one of the most commercialized nanomaterials with the production of 500 t of silver nanoparticles (AgNPs) per year [3].

Synthesis of AgNPs depends on the type of solvent used as well as on reducing and stabilizing agents, which determine whether it is a green or chemical/physical synthesis process. Usage of hazardous chemicals in chemical/physical synthesis methods has negative effects on humans, animals, plants and the environment due to toxicity, non-biodegradability and high cost. On the other hand, water is the most commonly used solvent in green AgNP synthesis, with additional advantages of lower energy consumption, simple performance, lower costs, utilization of non-toxic chemicals as reducing and stabilizing agents, which allow biocompatibility and *in vivo* applications [4-8]. AgNPs are widely applied because of their strong antibacterial activity against both Gram-positive and Gram-negative bacteria. They are used in commercial products, such as products for personal hygiene, detergents, cosmetics, sun lotions as well as in medical, textile and food products [9].

Reduction of Ag^+ ions can be achieved by various reduction agents among which plant extracts are becoming increasingly popular [10]. Plants can produce phytochemicals such as flavones, terpenoids, ketones, amides, aldehydes,

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proteins, carboxylic acids, glycosides *etc.* with different functional groups, which can reduce Ag^+ to Ag^0 acting also as capping agents [11-14]. In the present study, we have used aqueous extract of cultivated strawberry (*Fragaria x ananassa* Duch) leaves as a reducing and capping agent. Chemical composition of the extract was already analyzed and described in detail previously [15], suggesting the presence of phenolic compounds and flavonoids as potential reducing and capping agents. The usage of the obtained extract in stabilization of AgNPs-E was investigated along with the nanoparticle bio-activity (antioxidant and antibacterial) and the effects on viability of two epithelial-like cell lines.

2. EXPERIMENTAL

2. 1. Materials

Silver nitrate ($AgNO_3$) was obtained from RTB Bor Grupa, Serbia; 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, dimethyl sulfoxide (DMSO) and potassium-bromide (KBr) FT-IR grade from Sigma-Aldrich (Merck KgaA, Darmstadt, Germany). Nutrition agar plates from Torlak (Institute of Virology, Vaccines and Sera, Belgrade, Serbia); microorganisms *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 15313, *Bacillus luteus*, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* were in house strains; sterile cellulose discs 9 mm diameter are obtained from LLG Labware (Meckenheim, Germany).

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), stable glutamine, antibiotic-antimycotic solution, trypsin-EDTA and Hank's Balanced Salt Solution (HBSS) were purchased from Capricorn Scientific (Germany). 2-Propanol and ethanol (HPLC grade) were purchased from Fisher Scientific (Germany) while (4,5-dimethylthiazol-2-yl)--2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Carl Roth GmbH + Co. KG (Germany). For *in vitro* cytotoxicity study, two mammalian cell lines were used: MDCK (Madin-Darby canine kidney cells) and HeLa S3 (human cervical cancer cells), both obtained from ATCC (American Type Culture Collection, USA).

2. 2. Extraction

The extract is obtained according to the procedure described in detail earlier [15]. Dry leaves of cultivated strawberries of Mount Everest variety (climber) have been used in this work. The strawberries were cultivated in a greenhouse of 2.5 m height covered with polyethylene foil (0.15 mm thickness; Ginegar Plastic Products-Ltd.). The plant material was harvested at the beginning of May 2020 during the flowering period, shade-dried and ground in an electric mill (laboratory electric mill ''BRAUN AROMATIC KSM2'', Braun GmbH, Germany) just before analyzing.

Freshly ground and homogenized plant material (1 g) was extracted with 20 cm^3 of water for 120 min at 25 °C. After the extraction, the plant material was separated from the liquid extract by vacuum filtration on the Bichner's funnel. The obtained extract was kept in the refrigerator at +4 °C up to the analysis.

The yield of total extractive matter (TEM) was determined by using the SCALTEC SMO 01 (Scaltec Instruments, Germany) apparatus. The aliquot of 2 cm^3 of the extract was dried to a constant mass at 105 °C. The yield of TEM, calculated in relation to dry residue content, was 17 mg cm^{-3}.

2. 3. Synthesis of AgNPs-E

For the synthesis of AgNPs-E, the extract aliquot (2 cm^3) was mixed with 200 cm^3 of $AgNO_3$ solution (0.001 M) to the final concentration of the extract TEM in the mixture of 0.168 mg cm^{-3}. All solutions were prepared with twice re-distilled water. The synthesis was performed at room and boiling temperatures (reflux). In order to confirm the presence of AgNPs, the same aliquots of the samples were taken during the synthesis procedure, 20 times diluted and analyzed by UV-Vis spectroscopy. After the synthesis, all samples were dialyzed in Spectra/Por Biotech bags; cellulose ester; MWCO 100000 (Fisher Scientific, Germany). The bags were filled with 20 cm^3 of the AgNPs-E dispersion and immersed into redistilled water, slightly stirred on the magnetic stirrer. The portions of redistilled water were occasionally replaced; the absence of nitrate band in the UV-Vis spectra (~230 nm) of wastewater has been used as an indicator of nitrates removal. The dialysis process was carried out for about 48 h with occasional replacement of redistilled water. The

concentration of dialyzed $AgNP_S$-E was determined following the same procedure as for TEM determination (described in section 2.2.). After dry residue determination, the concentration of formed AgNPs-E was calculated. All further experiments were performed with dialyzed samples with AgNPs-E synthesized at RT for 144 h and at BT for 2 h.

2. 4. Spectroscopic methods

Spectroscopic characterization of synthesized AgNPs-E, stabilized by the strawberry leaves extract, was performed by UV-Visible and Fourier-transform infrared (FT-IR) spectroscopy.

2. 4. 1. UV-Vis spectroscopy

The UV-Vis spectra of 20-fold diluted reaction mixture were measured on a spectrophotometer Varian Cary-100 Konc (Varian, Australia), in the wavelength range of 200-800 nm; data processing was performed by software Cary WinUV Varian.

2. 4. 2. FT-IR spectroscopy

FT-IR spectra of solid samples were measured in the potassium-bromide pastilles on a BOMEM MB-100 (Hartmann & Braun, Canada) FT-IR spectrometer, equipped with a standard DTGS/KBr detector in the range of 4000-400 cm^{-1} with the resolution of 2 cm^{-1}. Data processing was performed by using a software package Win-Bomem EasyTM 3.01C Level II.

2. 5. DPPH-test

Ethanolic solution of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radicals (1.0 cm^3, 3×10^{-4} mol dm^{-3}) was added to 2.5 cm^3 of AgNPs-E solutions prepared at different concentrations (0.1, 0.085, 0.075, 0.05, 0.01, 0.005 and 0.001 mg cm^{-3}). Absorbance at 517 nm was measured after 20 min of incubation at room temperature in the dark ("sample"). The absorbance of a pure ethanolic solution of DPPH radical was also measured, diluted in an adequate proportion (1.0 cm^3 of DPPH radical, 3×10^{-4} mol dm^{-3}, diluted with 2.5 cm^3 of ethanol - "control"). The AgNPs-E solutions without DPPH radical added (2.5 cm^3 of AgNPs-E solutions diluted with 1.0 cm^3 of ethanol) served as the "blank". Free radical scavenging activity was calculated according to the equation [16,17]:

$$\text{DPPH radical scavenging activity} = 100 - \left[\left(A_S - A_B \right) \frac{100}{A_C} \right] \quad (1)$$

where:

A_S = absorbance of the „sample"

A_B = absorbance of the „blank"

A_C = absorbance of the „control"

2. 6. Antibacterial activity of AgNPs – E

Antibacterial activity of the AgNPs-E was determined by disc diffusion method against the following microorganisms: *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 15313, *Bacillus luteus*, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis*. Antibacterial activity was determined as previously described [18]. Bacterial suspensions were prepared by the direct colony suspension method. A few colonies of the overnight culture were directly suspended in sterile 0.85 % saline solution and the turbidity were adjusted by comparing with the 0.5 McFarland's standard. The initial bacterial suspensions contained about 10^8 CFU cm^{-3} and they were inoculated on the surface of nutrition agar plates. Sterile standard cellulose disks of 9 mm diameter were impregnated with 50 µL of AgNPs-E synthesized at room temperature (RT) and at boiling temperature (BT) in the total silver concentrations of 0.25 and 0.5 mg cm^{-3} and placed onto the surface of the inoculated plates. Plates were incubated at 37 °C for 24 h. The antibacterial activity was determined by measuring the inhibition zone diameter. All experiments were performed in triplicate.

2. 7. *In vitro* cytotoxicity testing

For *in vitro* cytotoxicity testing, two epithelial-like cell lines were used: MDCK (Madin-Darby canine kidney cells) and HeLa (human cervix cancer cells). These cell lines are the most commonly used as models for epithelial cells. Both cell lines were obtained from ATCC (American Type Culture Collection). Cells of both cell lines were cultured in DMEM supplemented with 10 % FBS, 2 mM stable glutamine and 1 % antibiotic-antimycotic solution (complete DMEM) at 37 °C in a humidified atmosphere containing 5 % CO_2.

2. 7. 1. Preparation of AgNPs-E for *in vitro* cytotoxicity testing

Silver nanoparticles synthesized at room (AgNPs-E-RT) and boiling (AgNPs-E-BT) temperatures were examined at different concentrations that were prepared by diluting the originally synthesized solution at concentration of 0.1 mg cm^{-3} in the cell culture medium (complete DMEM). The activity of AgNPs-E was examined in the solutions at the following concentrations: 0.05, 0.025, 0.015, 0.01, 0.005 and 0.002 mg cm^{-3}.

2. 7. 2. *In vitro* cytotoxicity testing

HeLa and MDCK cells were detached by using a trypsin-EDTA solution and cells were counted by using the Trypan Blue Dye Exclusion method. Cells were seeded in 96 well plates (Greiner Bio-One, Germany) at the density of 20,000 cells per well. After 24 h cell cultivation at standard culture conditions, 100 µL of AgNPs-E solutions (with concentrations that were twice as effective) were added to the cells (cultured in 100 µL of complete DMEM per well) at examined final concentrations as described in the section 2.7.1. Cells cultured in the standard cell culture medium (complete DMEM), without extracts (untreated cells) under the same conditions, were used as controls. Each concentration of AgNPs-E, as well as control, was tested in four to eight replicates. Cells were cultured with AgNPs-E-RT and AgNPs-E-BT for the next 24 h at the standard cell culture conditions.

2. 7. 3. MTT test

After 24 h of incubation, cell viability was assessed by the MTT test. Cells were first washed with phosphate buffer saline (PBS) followed by addition of 100 µL of the MTT solution (concentration 1 mg cm^{-3}). Cells were incubated with MTT for the next 3 h followed by dissolution of formed formazan crystals with 100 µL of 2-propanol per well. The amount of formed formazan is in direct correlation with the percentage of viable cells. The absorbance of dissolved formazan was measured by using a Multiskan Ascent Plate Reader (ThermoLab Systems, Finland) at the wavelength of 540 nm with the correction wavelength of 650 nm. The mean absorbance values were calculated for each examined concentration, as well as for the controls. The results are expressed as the percentage of cell viability that was calculated according to the formula:

$$\text{Cell viability} = \frac{A_S}{A_0} 100 \tag{2}$$

where:

A_S = absorbance of treated cells

A_0 = absorbance of untreated cells

For the control culture (untreated cells), cell viability was considered to be 100 %.

2. 7. 4. Statistical analysis

Statistically significant differences between the effects of AgNPs-E-RT and AgNPs-E-BT, as well as different cell lines, were analyzed by one-way analysis of variance (ANOVA) with absorbance values from at least two independent experiments. The value of $p < 0.05$ was considered as statistically significant.

3. RESULTS AND DISCUSSION

3. 1. UV-Vis characterization of synthesized AgNPs-E

The synthesis of nanoparticles at room (RT) and boiling (BT) temperature is illustrated by overlaid UV-Vis spectra, measured after certain time intervals and shown in Figure 1a and Figure 1b, respectively.

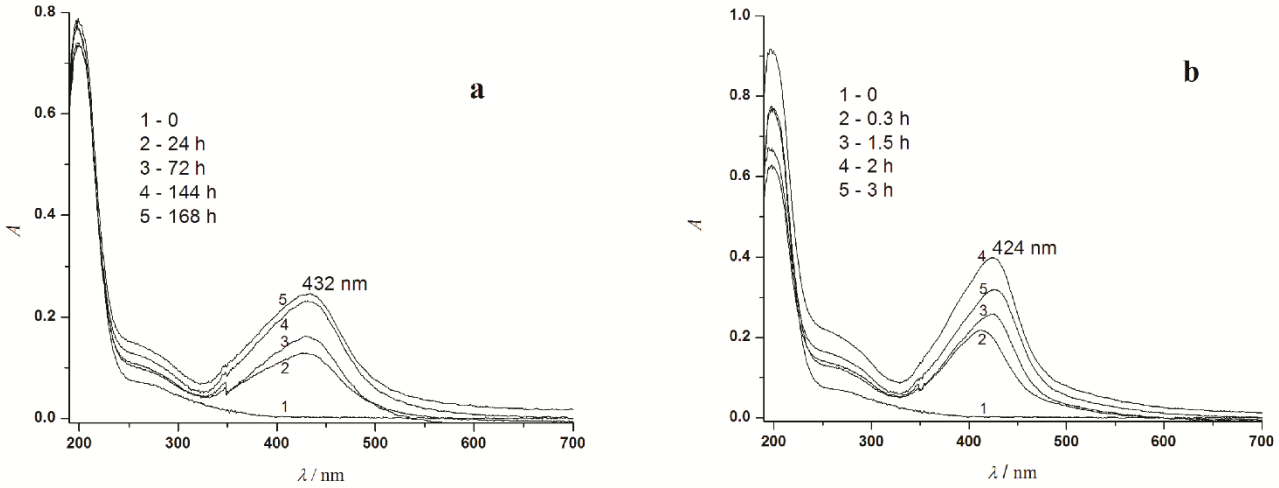

Figure 1. UV-Vis spectra of reaction mixtures - synthesis at room (a) and boiling temperature (b)

The effect of dialysis, *i.e.* removal of nitrate ions from the synthesized AgNPs-E solutions, is illustrated in Figure 2 (synthesis at BT). It should be emphasized that the reaction mixture for the synthesis at RT has shown very similar spectra (data not shown).

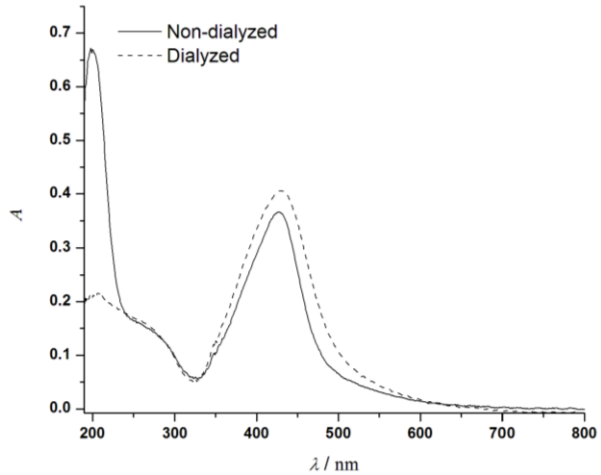

Figure 2. UV-Vis spectra of non-dialyzed and dialyzed solutions with AgNPs-E synthesized at boiling temperature

The stability of AgNPs-E at room temperature in dark was followed by UV-Vis spectroscopy (Fig. 3) for both investigated synthesis temperatures.

The synthesis of AgNPs-E, followed by changes of the reaction mixture colour, can be qualitatively and quantitatively monitored by the so-called Surface Plasmon Resonance (SPR) band at ~440 nm [8,19,20]. Increasing of the band intensity during synthesis (Fig. 1) is the evidence of the AgNP formation; the increase is proportional to the number of nanoparticles formed, while the band position is directly related to the particles size [19]. Based on the literature data that increasing diameter of nanoparticles from 10 to 100 nm leads to a shift of the absorption maxima from 400 to 500 nm [19], it is possible to assume that the synthesized particles are about 20 nm in diameter. The prediction of

nanoparticle size is additionally supported by already published results where AgNPs were synthesized and stabilized by an aqueous extract of *Fumaria officinalis* L. by the same procedure, where the nanoparticle size was determined as 21±1 nm (synthesized at RT) and 18±1 nm (synthesized at BT) by XRD and SEM methods [4]. It can be also concluded that AgNPs-E synthesis ends for about 140 h at RT and 2 h at BT (Fig. 1, a and b, respectively). Actually, at BT with the synthesis extension time to 3 h, the absorption peak is decreasing indicating lower AgNP concentration probably due to aggregation, which is promoted at higher temperatures. On the other hand, by comparing the peak intensities, it is implied that a higher AgNP-E concentration is obtained at BT (Fig. 1).

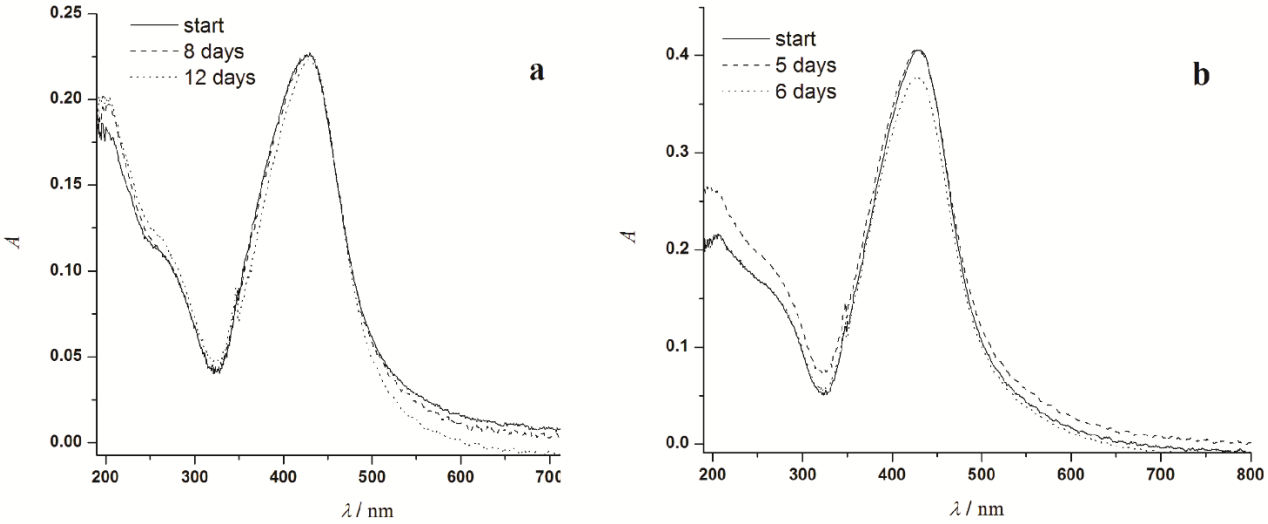

Figure 3. UV-Vis spectra of solutions with AgNPs-E synthesized at room (a) and boiling temperature (b) upon standing at room temperature in the dark – stability test

The effect of dialysis was monitored by changing of UV-Vis spectra configuration (Fig. 2). Reduction of the band (200 - 350 nm) intensity can be result of participation of some functional groups from the extract components in reduction and stabilization of formed nanoparticles. On the other hand, a significant decrease of absorbance in this range after dialysis is direct evidence that nitrates are removed from the samples. A negligible shift of the SPR band (Fig. 2) suggests that nanoparticles generally retain their size after dialysis and stability. In addition, it can be observed that the SPR band of dialyzed AgNPs-E did not shift upon staying at RT over 12 days suggesting high stability (Fig. 3).

3. 2. FT-IR spectroscopy

A comparative presentation of FT-IR spectra of the extract and solutions with AgNPs-E synthesized at boiling and room temperatures are shown in Figure 4.

The abundance of bands in the FT-IR spectra allows monitoring of the possible involvement of some functional groups from extract compounds in the reduction and stabilization of the formed AgNPs-E. The bands in the range of 3600-3200 cm^{-1} in the spectrum of the extract (Fig. 4, (1)) originate from valence ν_{-OH} (alcohols and phenols) and/or ν_{-NH} vibrations (amines, amides, peptides and proteins). The bands in the range of 2920 and 2850 cm^{-1} originate from ν_{-CH} vibrations. Bands around 1620 and 1530 cm^{-1} can be assigned to (NH)C=O vibrations (Amide I and II, $\nu_{C=O}$ and δ_{N-H}, respectively). The band at 1713 cm^{-1} originates from $\nu_{C=O}$ vibration of polyphenols. The vibrations of functional groups, which contain oxygen or nitrogen (NH, (NH)C=O, CNO, C-O-C and OH) are expected around 1240 cm^{-1}. These functional groups can participate in the silver reduction and stabilization of formed AgNPs-E. The functional groups of polyphenols, proteins, glycosides, alcohols, terpenoids, polysaccharides, polyols, *etc.*, – molecules, which are reducing and stabilizing agents [19,21], absorb in the range of 1200-1000 cm^{-1} (ν_{C-O}, ν_{C-O-C} and ν_{C-N} vibrations). The bands around 770 cm^{-1} originate from out-of-plane deformation vibrations of C-H groups, suggesting the presence of polyphenols [22,23].

By comparing the FT-IR spectrum of the extract with the spectra of solutions with synthesized AgNPs-E particles (Fig. 4), a significant reduction of the bands intensity in the latter spectra can be noticed.

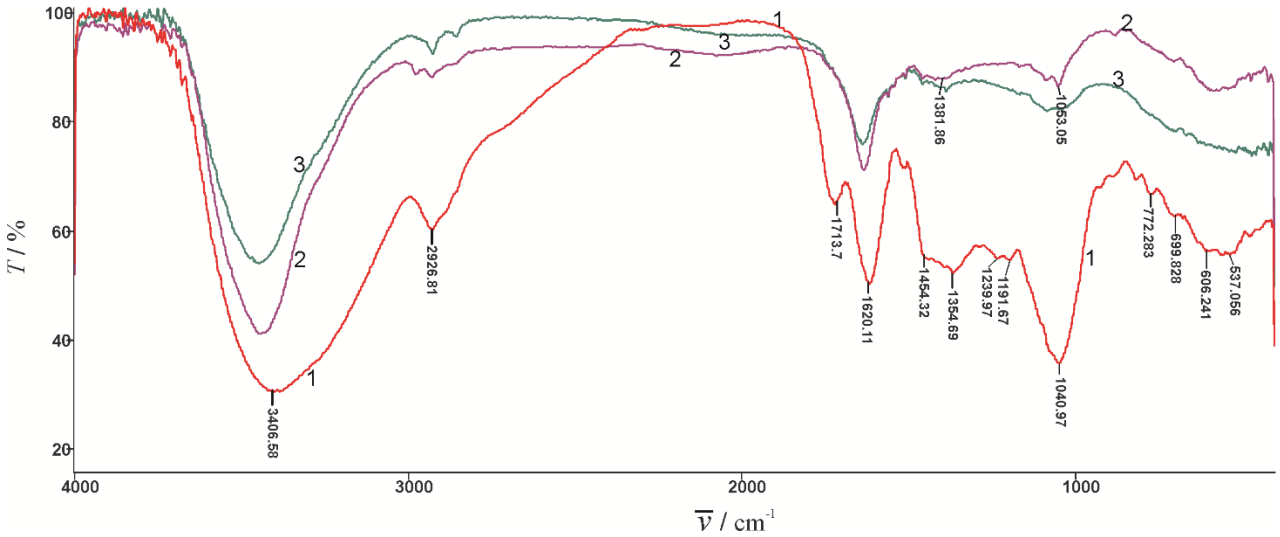

Figure 4. FTIR spectra of the extract (1) and solutions with AgNPs-E synthesized at boiling (2) and room temperature (3)

This reduction is especially noticed in the range of deformation vibrations at ~1380 cm^{-1} and ~1240 cm^{-1} (δ_{OH}). There is a slight shifting of the maximum at 1620 cm^{-1} to lower wavenumbers, while the band at 1713 cm^{-1} completely disappeared. In addition, a significant reduction of the bands intensity in the range of 1000-1350 cm^{-1}, suggests that molecules containing oxygen and nitrogen functional groups (NH, (NH)C=O, CNO, C-O-C and OH) participate in the reduction and stabilization of the formed nanoparticles.

3. 3. Antioxidant activity

The DPPH test is based on single (free) electron transfer between antioxidants and DPPH radicals [24-26]. Reduction of intensively purple DPPH radical to yellow hydrazine occurs during the reaction, which can be spectrophotometrically monitored by absorbance decreasing at 517 nm. A comparative review of DPPH radical neutralization by solution with AgNPs-E synthesized at room and boiling temperature is shown in Figure 5.

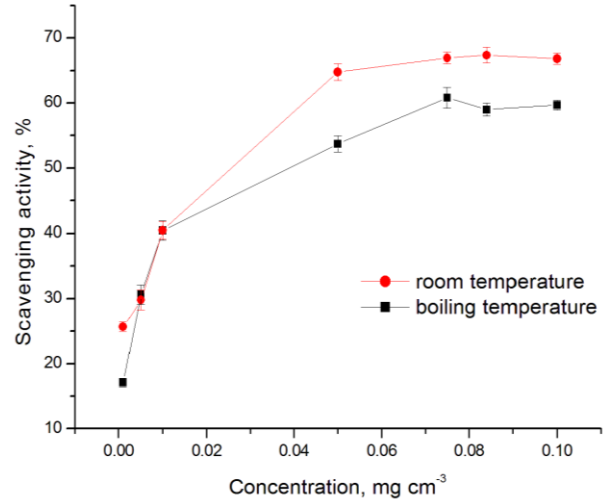

Figure 5. DPPH radical scavenging activity (%) as a function of the total silver concentration in solutions containing AgNPs-E synthesized at room temperature for 144 h and boiling temperature for 2 h

EC_{50} values represent the concentration of AgNPs-E needed to neutralize 50 % of the initial DPPH radical concentration. Based on the results represented in Figure 5 it can be concluded that the solutions with AgNPs-E synthesized at RT have a significantly higher ability to neutralize DPPH radicals (EC_{50} = 0.025 mg cm^{-3}) as compared to solutions with AgNPs-E synthesized at BT (EC_{50} = 0.039 mg cm^{-3}). With the increase of the sample concentrations above

0.01 mg cm^{-3}, the differences between antioxidant activities are more pronounced. A possible reason for this behaviour is a higher destruction of antioxidant molecules in the extract during the reaction at boiling temperature. It is possible that these components are degraded to a lesser extent during the reaction at room temperature due to significantly milder reaction conditions.

The extract used in this work has shown better antioxidant activity (0.013 mg cm^{-3}) [15] as compared to other strawberry leaves extracts: aqueous (0.487 mg cm^{-3}), methanolic (0.423 mg cm^{-3}) and ethanolic (0.655 mg cm^{-3}) extracts of strawberry tree leaves [27], as well as ethanolic extracts of three strawberry sorts: *Robus corchorifolius* (0.053 mg cm^{-3}), *Rubus parvifolius* (0.046 mg cm^{-3}) and *Duchesnea chrysantha* (0.067 mg cm^{-3}) [28]. The differences in antioxidant activity can be a consequence of extraction conditions, strawberry sort, and growing conditions, which candidates the strawberry sort and the extraction procedure applied in the present work as a material and technique of choice for further use. In the same time, the extract used in this work exhibited a lower EC_{50} value (0.013 mg cm^{-3}) [15], *i.e.* higher DPPH radical scavenging activity as compared to solutions after synthesis of AgNPs-E (Fig. 5). These results suggest that phenolic compounds, which are responsible for the antioxidant activity are probably engaged in reduction of silver ions and stabilization of formed nanoparticles *via* their functional groups and, to some extent, are "blocked" for DPPH radical neutralization, *i.e.* antioxidant activity. On the other hand, solutions with AgNPs-E synthesized in this work have shown higher antioxidant activities (lower EC_{50} values) as compared to solutions with nanoparticles synthesized using an aqueous leaf extract of *Atrocarpus altilis* (0.051 mg cm^{-3}) [29] and *Psidium guajava* L. (0.052 mg cm^{-3}) [30] as well as methanolic extracts of *Artemisia vulgaris* (> 0.2 mg cm^{-3}) [31].

3. 4. Antibacterial activity

Diameters of inhibition zones obtained for the examined microorganisms are given in Table 1, while representative photographs of agar plates are shown in Supplementary material (Fig. S1). Inhibition of growth has not been observed only for *B. cereus* bacteria.

Table 1. Antibacterial activity of solutions with AgNPs-E synthesized at room and boiling temperatures.

	Room temperature		Boiling temperature	
	AgNPs concentration, mg cm^{-3}			
	0.25	0.5	0.25	0.5
Bacterial strains	Average diameter of the inhibition zone ± SD, mm			
Gram positive bacteria				
B. cereus	-	-	-	-
B. luteus	15.3±0.6	18.3±0.6	12.7±0.6	14.0±0.0
B. subtilis	15.8±0.6	14.5±0.6	15.2±0.6	16.7±0.6
L. monocytogenes	17.3±0.6	18.5±0.5	12.3±0.6	15.7±1.2
S. aureus	17.3±0.6	17.8±0.8	13.3±0.6	15.0±0.0
Gram-negative bacteria				
P. vulgaris	-	-	-	-
K. pneumoniae	-	-	-	-
P. aeruginosa	-	-	-	-
E. coli	14.3±0.6	14.3±0.6	12.7±0.6	12.0±0.0

Antibacterial activity of AgNPs-E was examined against five strains of Gram-positive and four strains of Gram-negative bacteria. The activity of AgNPs-E against Gram-positive bacteria was much higher than against Gram-negative bacteria. Among analyzed Gram-negative bacteria, silver nanoparticles were active only against *E. coli*. Antibacterial activity against *E. coli* has been already confirmed for AgNPs stabilized with the extract of *Alternanthera dentate* [32], tea leaves [33], *Mentha piperita* [9] and *Aloe vera* [34]. The highest activity of AgNPs-E was observed against *L. monocytogenes* and *S. aureus*. Compared to the pure extract, synthesized AgNPs-E have a broader spectrum of antibacterial activity since pure extract was active only against *E. coli*, *S. aureus* and *P. vulgaris* [35]. Also, synthesized AgNPs-E have shown higher antibacterial activity as compared to AgNPs stabilized with carboxymethyl dextran applied at the same concentrations [36]. It can be noticed that the examined concentrations of AgNPs-E synthesized at RT had approximately same activity implied by similar sizes of inhibition zones. In addition, solution with AgNPs-E synthesized

at RT have shown slightly higher antibacterial activity compared to those with AgNPs synthesized at BT (Table 1), which is in accordance with the results of Nagajyothi and Lee [37]. The antibacterial effect of silver is based on several mechanisms including silver ions releasing from the nanoparticle surface, damaging of the cell membrane, disruption of electron transport, inhibition of DNA synthesis and oxidative stress. Due to oxidation, a silver oxide (Ag_2O) is formed on the nanoparticles' surface, which in reaction with the protons from the ambient medium forms free Ag^+ ion [38]. The silver ions are bonded by electrostatic interaction to cell membrane of the bacterium, increasing its permeability causing membrane destruction. Also, penetration of the ions inside the cell leads to cellular disorders, the impossibility of replication, and finally death [39-41]. The mechanism of antibacterial activity of synthesized AgNPs-E probably involves the holes in the cell membrane formation leading to their destruction [42,43]; the effect of silver on enzymes or protein synthesis in the cells of microorganisms, causing their metabolism disturbance, cannot be neglected, too [44,45]. According to the results, it can be assumed that antibacterial activity is a result of simultaneous activity of AgNPs and phenolic compounds originating from plant extract which support the approach of green synthesis process and usage of different plant for reduction and stabilization of AgNPs.

3. 5. *In vitro* cytotoxicity testing

Effects of different measured concentrations of AgNPs-E-RT and AgNPs-E-BT on the viability of two commonly used epithelial-like cell lines, HeLa and MDCK, were examined and assessed by the MTT test. The concentration-dependent effect was observed for both solutions with AgNPs-E-RT and AgNPs-E-BT in both examined cell lines. Cytotoxicity at higher examined concentrations (0.025 and 0.05 mg cm^{-3}) on both cell lines was noticed, although more pronounced on MDCK compared to HeLa cells. Statistically significant differences were not observed between the effects of solutions with AgNPs-E-RT and AgNPs-E-BT on the HeLa cell viability at any examined concentration (Fig. 6a).

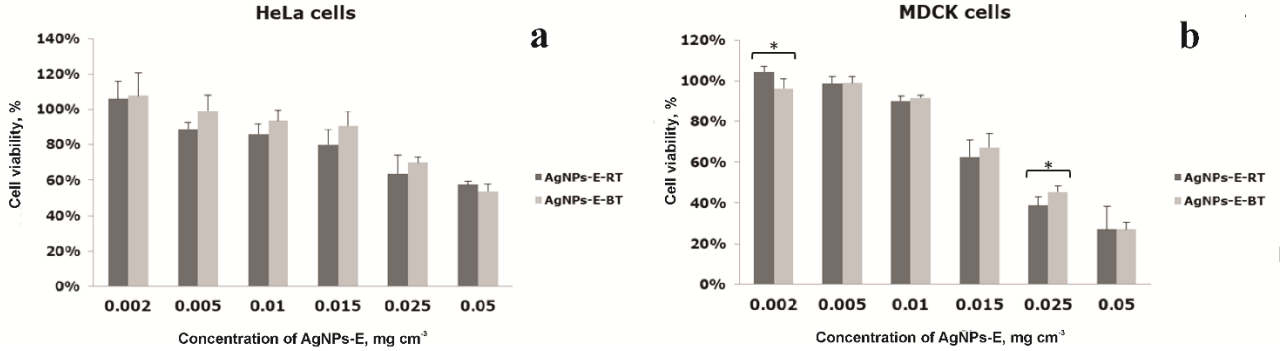

Figure 6. Viability of HeLa cells (a) and MDCK cells (b) after 24 h incubation with AgNPs-E-RT and AgNPs-E-BT solutions, evaluated by the MTT test (*) $p < 0.05$

However, when the effects of solutions with AgNPs-E were examined on MDCK cells, a statistically significant difference in the effect on cell viability was observed between AgNPs-E-RT and AgNPs-E-BT at two examined concentrations, 0.002 and 0.025 mg cm^{-3} (Fig. 6b). Significant differences in the effects at higher examined concentrations of solutions with AgNPs-E-RT and AgNPs-E-BT (from 0.015 to 0.05 mg cm^{-3}) on cell viability were noticed when HeLa and MDCK cells were compared.

More pronounced cytotoxicity (decreased cell viability) that was observed at concentrations in the range 0.015 to 0.05 mg cm^{-3} for both AgNPs-E-RT and AgNPs-E-BT on MDCK cells, compared to HeLa cells, can be explained by the differences between those two cell lines in terms of their origin, cellular characteristics and specific functions that they perform. This result leads us to the conclusion that MDCK cells were more sensitive to the nanoparticles than HeLa cells.

The presented results are comparable with the results recently published by other researchers [7,46] suggesting that AgNPs synthesized with the use of the strawberry leaf extract have a potential as the ingredient in cosmetic preparations for the treatment of various skin conditions [47,48].

4. CONCLUSION

Silver nanoparticles stabilized by an aqueous extract of grown strawberry leaves have shown high stability at room temperature. FT-IR analyses indicates that molecules from the extract, containing oxygen and nitrogen functional groups, participate in the reduction and stabilization of formed nanoparticles. In the same time, the extracts with the obtained nanoparticles have shown antioxidant (DPPH test) and antibacterial activity (disc diffusion method). A concentration-dependent effect of nanoparticles on cell viability was observed in both examined cell lines with the more pronounced cytotoxicity on MDCK cells as compared to HeLa cells, at higher examined concentrations. Based on the obtained results, it can be concluded that silver nanoparticles, stabilized by the aqueous extract of strawberry leaves, can be used under certain conditions as a base for further production of pharmaceutical preparations intended for the use as dermocosmetics.

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REFERENCES

[1] Ramsden J. Essentials of Nanotechnology, What is Nanotechnology, Ventus Publishing ApS; 2009.

[2] Pramanik N, Bhattacharyya A, Kundu PP. Spectroscopic analysis and catalytic application of biopolymer capped silver nanoparticle, an effective antimicrobial agent. *J Appl Polym Sci*. 2015; 132(8): 41495.

[3] Fatal T, Taştan P, Tüzün BS, Ozyazici M, Kivcak B. Synthesis characterization and studies on antioxidant activity of silver nanoparticles using *Asphodelus aestivus* Brot. aerial part extract. *S Afr J Bot*. 2017; 112: 346–353.

[4] Cakić M, Glišić S, Cvetković D, Cvetinov M, Stanojević Lj, Danilović B, Cakić K. Green synthesis, characterization and antibacterial activity of silver nanoparticles produced from *Fumaria officinalis* L. plant extract. *Colloid J*. 2018; 80(6): 803–813.

[5] Venkatadri B, Shanparvish E, Rameshkumar MR, Arasu MV, Al-Dhabi NA, Ponnusamy VK, Agastian P. Green synthesis of silver nanoparticles using aqueous rhizome extract of *Zingiber officinale* and *Curcuma longa*: *In-vitro* anti-cancer potential on human colon carcinoma HT-29 cells. *Saudi J Biol Sci*. 2020; 27: 2980–2986.

[6] Das CGA, Kumar VG, Dhas TS, Karthick V, Govindaraju K, Joselin JM, Baalamurugan J. Antibacterial activity of silver nanoparticles (biosynthesis): A short review on recent advances. *Biocatal Agric Biotechnol*. 2020; 27: 101593.

[7] MubarakAli D, Thajuddin N, Jeganathan K, Gunasekaran M. Plant extract mediated synthesis of silver and gold nanoparticles and its antibacterial activity against clinically isolated pathogens. *Colloids Surf B*. 2011; 85(2): 360–365.

[8] Amooaghaie R, Saeri MR, Azizi M. Synthesis, characterization and biocompatibility of silver nanoparticles synthesized from *Nigella sativa* leaf extract in comparison with chemical silver nanoparticles. *Ecotoxicol Environ Saf*. 2015; 120: 400–408.

[9] Lima RD, Seabra AB, Durán N. Silver nanoparticles: a brief review of cytotoxicity and genotoxicity of chemically and biogenically synthesized nanoparticles. *J Appl Toxicol*. 2012; 32(11): 867–879.

[10] Vijayaraghavan K, Nalini SPK. Biotemplates in the green synthesis of silver nanoparticles. *Biotechnol J*. 2010; 5: 1098–1110.

[11] Shankar SS, Rai A, Ahmad A, Sastry M. Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using neem (*Azadirachta indica*) leaf broth. *J Colloid Interface Sci.* 2004; 275(2): 496–502.

[12] Sherin L, Sohail A, Amjad US, Mustafa M, Jabeen R, Ul-Hamid A. Facile green synthesis of silver nanoparticles using *Terminalia bellerica* kernel extract for catalytic reduction of anthropogenic water pollutants. *Colloid Interface Sci Commun*. 2020; 37: 100276.

[13] Nestor ARV, Mendieta VS, Lopez MAC, Espinosa RMG, Lopez MAC, Alatorre JAA. Solventless synthesis and optical properties of Au and Ag nanoparticles using *Camellia sinensis* extract. *Mater Lett*. 2008; 62: 3103–3105.

[14] Aragão AP, Oliveira TM, Quelemes PV, Perfeito MLG, Araújo MC, Santiago JAS, Cardoso VS, Quaresma P, Leite JRA, Silva DA. Green synthesis of silver nanoparticles using the seaweed *Gracilaria Birdiae* and their antibacterial activity. *Arab J Chem.* 2019; 12(8): 4182–4188.

[15] Cvetković DJ, Stanojević LjP, Stanković MZ, Cakić MD, Savić SR, Miljković MD. Antioxidant activity of strawberry (*Fragaria × ananassa* Duch.) leaves. *Sep Sci Technol*. 2017; 52: 1039–1051.

[16] Cvetković D, Marković D. UV-effects on antioxidant activity of selected carotenoids in the presence of lecithin estimated by DPPH test. *J Serb Chem Soc*. 2008; 73(11): 1051–1061.

[17] Stanojević LjP, Zdravković AS, Stanković MZ, Cakić MD, Nikolić VD, Ilić DP. Antioksidativna aktivnost vodeno-etanolnih ekstrakata iz lista koprive (*Urtica dioica* L.). *Savremene tehnol*ogije. 2013; 2(1):51–59. (In Serbian)

[18] Veličković JM, Kostić DA, Stojanović GS, Mitić SS, Mitić MN, Ranđelović SS, Đorđević AS. Phenolic composition, antioxidant and antimicrobial activity of the extracts from *Prunus spinosa* L. fruit. *Hem. ind*. 2014; 68(3): 297–303. (In Serbian)

[19] Bednar N. Synthesis of metallic nanoparticles in electrolyte-plasma interface [dissertation]. Novi Sad: University of Novi Sad, Serbia; 2014.

[20] Sabri MA, Umer A, Awan GH, Hassan MF, Hasnain A. Selection of suistable biological method for the synthesis of silver nanoparticles. *Nanomater nanotechnol*. 2016; 6(29): 1–20.

[21] Ebrahiminezhad A, Barzegar Y, Ghasemi Y, Berenjian A. Green synthesis and characterization of silver nanoparticles using *Alcearosea* flower extract as a new generation of antimicrobials. *Chem Ind Chem Eng Q*. 2017; 23(1): 31–37.

[22] Ivanov IG, Vracheva VZ, Marchev AS, Petkova NT, Aneva IY, Danev PP, Georgiev VG. Antioxidant activites and phenolic compounds in *Bulgarien Fumaria* species. *Int J Curr Microbiol App Sci*. 2014; 3(2): 296–306.

[23] Kumar V, Yadov SK. Plant-mediated synthesis of silver and gold nanoparticles and their applications. *J Chem Technol Biotechnol*. 2009; 84: 151–157.

[24] Iacopini P, Baldi M, Storchi P, Sebastiani L. Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, *in vitro* antioxidant activity and interactions. *J Food Compos Anal.* 2008; 21(8): 589–598.

[25] Ndhlala AR, Moyo M, Staden JV. Natural antioxidants: Fascinating or mythical biomolecules. *Molecules.* 2010; 15(10): 6905–6930.

[26] Pouillot A, Polla LL, Tacchini P, Neequaye A, Polla A, Polla B. Natural antioxidants and their effects on the skin, *John Wiley Sons Inc*. 2011; 239–257.

[27] Orak HH, Yagar H, Isbilir SS, Demirci AS, Gümüş T, Ekinci N. Evaluation of antioxidant and antimicrobial potential of strawberry tree (*Arbutus Unedo* L.) leaf. *Food Sci Biotechnol*. 2011; 20(5): 1249–1256.

[28] Seleshe S, Lee JS, Lee S, Lee HJ, Kim GR, Yeo J, Kim JY, Kang SN. Evaluation of antioxidant and antimicrobial activities of ethanol extracts of three kinds of strawberries. *Prev Nutr Food Sci*. 2017; 22(3): 203–210.

[29] Ravichandran V, Vasanthi S, Shalini S, Shah SAA, Harish R. Green synthesis of silver nanoparticles using *Atrocarpus altilis* leaf extract and the study of their antimicrobial and antioxidant activity. *Mater Lett*. 2016; 180: 264–267.

[30] Wang L, Wu Y, Xie J, Wu S, Wu Z. Characterization, antioxidant and antimicrobial activities of green synthesized silver nanoparticles from *Psidium guajava* L. leaf aqueous extracts. *Mater Sci Eng C*. 2018; 86: 1–8.

[31] Rasheed T, Bilal M, Iqbal HMN, Li C. Green biosynthesis of silver nanoparticles using leaves extract of *Artemisia vulgaris* and their potential biomedical applications. *Colloids Surf B*. 2017; 158: 408–415.

[32] Kumar DA, Palanichamy V, Roopan SM. Green synthesis of silver nanoparticles using *Alternanthera dentata* leaf extract at room temperature and their antimicrobial activity. *Spectrochim Acta, Part A*. 2014; 127: 168–171.

[33] Nabikhan A, Kandasamy K, Raj A, Alikunhi NM. Synthesis of antimicrobial silver nanoparticles by callus and leaf extracts from saltmarsh plant, *Sesuvium portulacastrum* L. *Colloids Surf B*. 2010; 79(2): 488–493.

[34] Zhang Y, Yang D, Kong Y, Wang X, Pandoli O, Gao G. Synergetic antibacterial effects of silver nanoparticles *Aloe Vera* prepared via a green method. *Nano Biomed Eng*. 2010; 2(4): 252–257.

[35] Stanojević Lj, Cvetković D, Danilović B, Cakić M, Stanojević D. Antioxidant and antimicrobial activity of aqueous extracts from cultivated strawberry (*Fragaria x ananassa* Duch.) leaves from Serbia, 24th Congress of Chemists and Technologists of Macedonia, Ohrid, Republic of Macedonia; 2016, p. 212.

[36] Glišić S, Cakić M, Nikolić G, Danilović B. Synthesis, characterization and antimicrobial activity of carboxymethyl dextrane stabilized silver nanoparticles. *J Mol Struct*. 2015; 1084: 345–351.

[37] Nagajyothi PC, Lee KD. Synthesis of plant-mediated silver nanoparticles using *Dioscorea batatas* rhizome extract and evaluation of their antimicrobial activities. *J Nanomaterials*. 2011; 1–7.

[38] Xiu ZM, Ma J, Alvarez PJJ. Differential Effect of Common Ligands and Molecular Oxygen on Antimicrobial Activity of Silver Nanoparticles versus Silver Ions. *Environ Sci Technol*. 2011; 45(20): 9003–9008.

[39] Li WR, Xie XB, Shi QS, Duan SS, Ouyang YS, Chen YB. Antibacterial effect of silver nanoparticles on *Staphylococcus aureus*. *BioMetals.* 2011; 24(1): 135–141.

[40] Barani H, Montazer M, Samadi N, Toliyat T. In situ synthesis of nano silver/lecithin on wool: Enhancing nanoparticles diffusion. *Colloids Surf B*. 2012; 92: 9–15.

[41] Gutierrez FM, Thi EP, Silverman JM, Oliveira D, Camargo C, Svensson SL, Hoek AV, Sánchez EM, Reiner NE, Gaynor EC, Pryzdial EL, Conway EM, Orrantia E, Ruiz F, Av-Gay Y, Bach H. Antibacterial activity, inflammatory response, coagulation and cytotoxicity effects of silver nanoparticles. *Nanomedicine*. 2012; 8: 328–336.

[42] Shrivastava S, Bera T, Roy A, Singh G, Ramachandrarao P, Dash D. Characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology*. 2007; 18: 225103–225112.

[43] Tamboli DP, Lee DS. Mechanistic antimicrobial approach of extracellularly-synthesized silver nanoparticles against Gram positive and Gram negative bacteria. *J Hazard Mater*. 2013; 260: 878–884.

[44] Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J Biomed Mater Res*. 2000; 52(4): 662–668.

[45] Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra SY, Mclaughlin MJ, Lead JR. Nanomaterials in the environment: behavior, fate, bioavailability and effects. *Environ Toxicol Chem*. 2008; 27(9): 1825–1851.

[46] Hepokur C, Kariper İA, Mısır S, Ay E, Tunoğlu S, Ersez MS, Zeybek U, Kuruca SE, Yaylım İ. Silver nanoparticle/capecitabine for breast cancer cell treatment, *Toxicol In Vitro*. 2019; 61: 104600.

[47] Parveena A, Kulkarni N, Yalagatti M, Abbaraju V, Deshpande R. *In vivo* efficacy of biocompatible silver nanoparticles cream for empirical wound healing. *J Tissue Viability*. 2018; 27: 257–261.

[48] Prociak JP, Grabowska A, Chwastowski J, Majka TM, Banach M. Safety of the application of nanosilver and nanogold in topical cosmetic preparations. *Colloids Surf B*. 2019; 183: 110416.

САЖЕТАК

Водени екстракт листова гајене јагоде (Fragaria x ananassa Duch.) као стабилизирајући агенс у синтези биоактивних наночестица сребра

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(Научни рад)

Циљ рада је провера активности воденог екстракта листова гајене јагоде (*Fragaria x ananassa* Duch.) у стабилизацији наночестица сребра (AgNPs-E) синтетисаних на собној температури и температури кључања. Синтеза и стабилност *AgNPs-E* су праћени UV-видљивом спектроскопијом чиме је потврђена велика стабилност наночестица на собној температури у мраку. Молекули екстракта који садрже функционалне групе са кисеоником и азотом (NH, (NH)C=O, CNO, C-O-C и OH) учествују у редукцији и стабилизацији *AgNPs-E* што је показано техником инфрацрвене спектроскопије са Фуријеовом трансформацијом (FTIR). Узорци наночестица сребра синтетисани на собној температури показали су већу антиоксидативну активност у поређењу са узорцима у којима су наночестице синтетисане на температури кључања (EC_{50} вредности 0,025 и 0,039 mg cm^{-3}, редом) што је утврђено *DPPH* тестом. Такође, *AgNPs-E* синтетисане на собној температури показале су већу антибактеријску активност против *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus subtilis* и *Bacillus luteus*. Испитивање AgNPs-E на HeLa и MDCK ћелијским линијама показало је концентрациону зависност и ефекат на вијабилност ћелија који је специфичан за ћелијску линију, што је процењено *MTT* тестом. Добијени резултати показују да се синтетисане наночестице сребра могу користити као сировина у производњи фармацеутских препарата за потенцијалну примену на кожи.

Кључне речи: AgNPs; биљни екстракт; зелена синтеза; антимикробна активност; антиканцер активност.