Cytotoxic and antimicrobial effect of biosynthesized silver nanoparticles using the fruit extract of *Ribes nigrum*

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Abstract
The present study reveals the efficiency of the fruit extract of *Ribes nigrum* in the green synthesis of silver nanoparticles (Ag-NPs). Biosynthesized Ag-NPs were characterized by UV-vis, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and atomic force microscopy (AFM). The nanoparticles were found to be 5–10 nm. In some places, the particles were agglomerated. The nanoparticles showed strong bactericidal activity and fungicidal activity against dermatophytes *Trichophyton rubrum* ATCC 28188. Moreover, the A549 and CCD39Lu cells under the influence of the highest concentration of nanoparticles synthesized using the fruit extract of *Ribes nigrum* showed the maximum mortality. Also, the results indicate that Ag-NPs synthesized using the fruit extract of *Ribes nigrum* exhibit efficiency in therapy of human non-small cell lung cancer A549.

Keywords: biosynthesis, silver nanoparticles, antimicrobial activity, anticancer activity
Classification numbers: 2.03, 4.02, 5.08

1. Introduction

Ag-NPs have generated wide interest as they have numerous applications in different fields, such as dentistry, clothing, catalysis, mirrors, optics, photography, electronics, and the food industry [1]. The most widely used and known applications of Ag-NPs occur in the medical industry, which includes ointments and creams to prevent infections of burns and open wounds [2]. Ag-NPs show an inhibitory effect on many bacterial strains and microorganisms [3]. Apart from their antimicrobial properties, nowadays biogenic Ag-NPs have been explored in the field of nano-oncology to recognize their capability for the expansion of cancer treatment [4]. However, the cytotoxicity of Ag-NPs depends on their shape, size, surface chemistry, etc [5].

Biological methods for synthesizing nanoparticles have been suggested as possible eco-friendly alternatives to chemical and physical methods. The use of plants in the synthesis of nanoparticles is quite novel and leads to truly green chemistry, as it is cost-effective and environmentally friendly. Moreover, this method does not require the use of high pressure, energy, temperature, and toxic chemicals [6]. The biomolecules present in the biological sources have reducing properties that can reduce metal ions (M⁺) to zero-valent (MO) metal form and stabilize them. What is more, the use of plant extract can hinder the aggregation of the synthesized metal nanoparticles and control their particle sizes [7]. Many research articles reported the synthesis of Ag-NPs using plant...
extracts such as Citrullus colocynthis [8], Alternanthera dentata [9], Rhododendron dauricum [10], Acalypha indica [11], Artemisia nilagirica [12], Boswellia ovalifoliolata [13], Nelumbo nucifera [14], Desmodium triflorum [15], Hibiscus cannabinus [16], Sesuvium portulacastrum [17], Stigmaphyllon littorale [18], Trachyspermum coticum [19] and Rosa rugosa [20].

Ribes nigrum is a widely cultivated plant all over the world, mainly for its high-value berries possessing unique flavor and containing various bioactive constituents [21]. The major components are the ascorbic acid, a micronutrient which is essential for human health, and polyphenols with anthocyanins as the most abundant [22]. Within anthocyanins, cyanidin-3-rutinoside and delphinidin-3-rutinoside predominated, followed by cyanidin-3-glucoside and delphinidin-3-glucoside. Figure 1 presents the chemical structures of cyanidin-3-rutinoside and delphinidin-3-rutinoside. The flavonol glycosides of quercetin, myricetin and kaempferol are only present in much lower concentrations [23].

Due to the fact that lung cancer—a highly invasive, rapidly metastasizing and prevalent cancer—is the top killer cancer in both men and women [24], in this study, the synthesized Ag-NPs were investigated in terms of their anticancer activity. As it is known, it is an extraordinary challenge to discover anticancer substances that will kill or disable only tumor cells without undue toxicity [25]. As Ribes nigrum is rich in biologically active compounds, its powdered fruits were used for the synthesis of Ag-NPs. The obtained Ag-NPs were characterized and assessed in terms of their antibacterial and cytotoxic activity.

2. Materials and methods

2.1. Synthesis of Ag-NPs

Fresh and healthy Ribes nigrum fruits were collected from Wielkopolska region (Poland) (52.1714 °N, 17.5218 °E) in July 2015. The fruits were washed with deionized water, dried in a dark place and powdered. The reducing agent for AgNO₃ was prepared by taking 5 g powder of Ribes nigrum fruits and boiling them in 250 ml of sterile milliQ water for the period of 45 min in the temperature of 90 °C. 20 ml extract was mixed with 10 ml of AgNO₃ (1 mM). The solution was stirred 24 h in darkness. The reaction was carried out at 25 °C. The biosynthesis of Ag-NPs was monitored by sampling the reaction mixture at regular intervals (24 h) and the absorption maximum was scanned by UV-vis spectra at the wavelength of 350–800 nm. Moreover, the absorbance measurements were performed after 48, 72, 96 and 120 h after preparing the solution of Ag-NPs.

2.1.1. Characterization of Ag-NPs. The UV-vis absorption spectrum of Ag-NPs was performed in the spectrophotometer Cary E 500. The reduction of silver ions was monitored by UV-vis spectrum of the solution between the ranges of 350–800 nm. The Ag-NPs were studied with transmission electron microscopy. TEM measurements were performed on the JEM 1200 EXII, operating at 80 kV. The surface morphology of the prepared sample was also examined using an AFM, Agilent 5500. The sizes of Ag-NPs were analyzed using scanning electron microscopy (SEM) Quanta FEG 250 (FEI). The FTIR analysis of the synthesized Ag-NPs was carried out by using Perkin Elmer Spectrum 1000, in attenuated total reflection mode and using the spectral range of 4000 – 380 cm⁻¹ with the resolution of 4 cm⁻¹.

2.1.2. Antimicrobial activity of Ag-NPs. The antimicrobial activity was evaluated against reference strains of bacteria (Staphylococcus aureus ATCC 4163, Pseudomonas aeruginosa ATCC 6749, Escherichia coli ATCC 25922), yeast (Candida albicans ATCC 10231), filamentous fungi (Aspergillus niger ATCC 16404) and dermatophytes (Trichophyton rubrum ATCC 28188), as well as clinical isolates of Pseudomonas aeruginosa K1, Staphylococcus aureus K1 and Candida albicans K1. A macrodilution broth method was used to determine the minimal inhibitory concentrations (MIC) according to EUCAST references with modifications using: Mueller-Hinton broth (MHB) for bacteria, and Sabouraud dextrose broth (SDB) for fungi. The MIC was defined as the lowest concentration at which visible growth was not observed. Minimal bactericidal (MBC) and fungicidal (MFC) concentrations were determined as an extension of the MIC tests. The MBC/MFC was defined as the lowest concentration at which no growth was observed. Amikacin and nystatin were used as standard substances. Simultaneously, assessment
of *Ribes nigrum* fruit water extract effect on microbes used in the study was carried out using the same method.

### 2.1.3. Evaluation of cell proliferative activity of synthesized Ag-NPs.

#### 2.1.3.1. Established cell lines.

In vitro studies of the influence of Ag-NPs synthesized from the extract of *Ribes nigrum* were conducted using two established human cell lines. One was a non-malignant line of fibroblasts CCD-39Lu (ATCC CRL-1498) isolated from lungs, and the second was adherent epithelial non-small cell lung cancer cell line A549 (ATCC BCA-185). Cells were cultured on plastic 24-well plates with flat-bottom (TC-PLATE 24 well, Greiner) in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). For protection against microbiological contamination, cells were grown with the addition of 1% antibiotic-antimycotic mixture (10 000 U penicillin, 10 mg·ml⁻¹ streptomycin, and 25 mg·ml⁻¹ amphotericin B). Cells were grown in an incubator at 37°C in a humidified atmosphere with 5% CO₂ saturation.

#### 2.1.3.2. Assessment of the cell cycle with the use of propidium iodide.

Cells were cultured in the presence of appropriately concentrated solutions of Ag-NP for 24, 48 and 72 h. After subsequent periods of time, the cell cycles were evaluated. The assessment was focused on the calculation of the percentage of cells in the S phase, in the G2/M phase, and dead cells. The basis for testing the proliferative activity was the percentage of cells in S-phase of the cell cycle. The determination of the percentage of cells was based on the measurement of mean fluorescence intensity (MFI) emitted by propidium iodide (PI). It is fluorochrome intercalating into the DNA of replicating cells. The intensity of the fluorescence emitted by PI in the S-phase of the cell cycle is equivalent to the proliferative activity. The arrest of cell cycle and increase in the percentage of cells in G2/M-phase are often associated with a boosting of apoptosis pathway in cells. The assay procedure was carried out according to the previously described protocol [26]. Briefly, cells were transferred to culture plates, in the concentration of 4 × 10⁴ cells/well. When the cells adhered to the plate surface, after 24 h culture media were changed and the Ag-NP solutions in freshly prepared media were added in quantities: 1 µM, 10 µM, and 100 µM. Negative controls were grown in culture media without test substance. Cells were incubated in triplicates for 24, 48, and 72 h. When the cultures were finished, cells were harvested from plates and resuspended in 600 µl of cold permeabilization buffer containing 1% saponin (Sigma), RPMI-1640 medium supplemented with 10% FBS. After 30 min incubation, cells were centrifuged at 400 g for 5 min at 4°C. Next, cell pellets were resuspended in cold PBS containing 10 mg·ml⁻¹ propidium iodide (Sigma) and 100 U ml⁻¹ RNase enzyme (Boehringer Mannheim) and incubated for 30 min at 4°C protected from light. Prepared samples were acquired with the use of flow cytometer FACS Canto (Becton Dickinson). Analyses of percentages of cells in the cell cycles were calculated using FACSDiva software (Becton Dickinson).

### 2.1.4. Statistical analysis.

The result of antimicrobial activity of prepared Ag-NPs extract of *Ribes nigrum* was analyzed using Kruskal-Wallis test. *p*-value < 0.05 was considered to be significant. The software STATISTICA was employed for the statistical analysis.

### 3. Results and discussion

#### 3.1. UV-vis analysis of Ag-NPs

Figure 2 presents the UV-vis spectra of the synthesized Ag-NPs using extract of *Ribes nigrum* fruits. The absorbance was read at 48 h, 72 h, 96 h and 120 h after preparing the solution of Ag-NPs. The number and frequency of measurements stemmed from the fact that the time required for the complete reduction of metal ions during the biosynthesis of metal nanoparticles using bacteria and fungi is from 24 to 120 h [27]. The absorption spectra of the reaction media have the absorbance at 450 nm that has confirmed the presence of Ag-NPs. It was noted that absorbance increased with time. The increase in intensity could be caused by the increasing number of nanoparticles formed as a result of the reduction of silver ions presented in the aqueous solution [28].
3.2. FTIR analysis of Ag-NPs

As it is known, Fourier transform infrared spectroscopy (FTIR) has become an important tool in understanding the involvement of functional groups in relation between metal particles and biomolecules. Also, FTIR analysis was used for the characterization of the synthesized Ag-NPs (figure 3). Clear and broad absorbance bands were observed at 3311 cm\(^{-1}\), 2139 cm\(^{-1}\), 1634 cm\(^{-1}\), and 423 cm\(^{-1}\). A broad peak at 3311 cm\(^{-1}\) shows O – H stretching due to alcoholic group. The intense peaks were observed at 2139 cm\(^{-1}\) and 1634 cm\(^{-1}\), attributed to C = C stretch in aromatic ring and C = O stretch in polyphenols present for the fruits of Ribes nigrum. The observed peaks have considered major functional groups in different chemical classes such as polyphenols and anthocyanins. It is confirmed, among others, by studies on the content of biologically active substances in Ribes nigrum. Due to such properties of the compounds, the fruit extract of Ribes nigrum was used in order to synthesize Ag-NPs.

According to the literature, Ribes nigrum fruit is rich in antioxidants, such as vitamin C [29] and phenolic compounds, including flavonoids such as anthocyanins, flavonols, phenolic acids, flavan-3-ols, and tannins, which have health-promoting properties [30–33]. The content of polyphenols and anthocyanins is directly related to the antioxidant properties. Apart from anthocyanins, blackcurrant fruit contains their colorless precursors—proanthocyanidins and polycatechins, which also show antioxidant properties [34]. Blackcurrant also contains 18 different phenolic acids, mostly derivatives of hydroxycinnamic acid, hydroxybenzoic acid, p-hydroxyphenolacetic acid and p-hydroxyphenyllactic acid. Moreover, blackcurrant contains sinapic acid, which was not identified in any other berry fruits. The main free polyphenolic acids found in blackcurrant are m-coumaric acid, ferulic acid, and p-hydroxyphenolacetic acid [35].

According to the literature, many studies have demonstrated the excellent antioxidant activity of blackcurrant extract and its health benefits, including anticarcinogenic activity [36].

Snyder states [37] that water has the highest polarity index, followed by methanol, acetone and acetic acid. Anthocyanins are regarded as polar compounds due to the fact that their particles contain hydroxyl, carboxyl and methoxy groups bound to an aromatic ring. Therefore, they dissolve much better in polar solvents than in non-polar ones [38]. For this reason, this work used water extract. Furthermore, the literature has shown that, for example, the application of water extraction solvents with acid and the application of water and methanol extraction solvents with acetic acid result in a similar content of anthocyanins in the Ribes nigrum fruit extracts [39].

3.3. TEM analysis of Ag-NPs

The TEM images of synthesized Ag-NPs using the water extract of Ribes nigrum show that the particles are monodispersed and spherical in shape. The size range of the nanoparticles was found to be 5–10 nm. Moreover, transmission electron microscopy images indicate good crystallinity of the synthesized nanoparticles. Figure 4 presents TEM images (a) and (b) of synthesized Ag-NPs using fruit extract of Ribes nigrum with the scale bar of 100 nm.

Figure 3. FTIR spectrum of synthesized Ag-NPs using extract of Ribes nigrum fruits.

Figure 4. TEM images (a) and (b) of synthesized Ag-NPs using fruit extract of Ribes nigrum with the scale bar of 100 nm.
3.4. SEM analysis of Ag-NPs

Imaging by means of SEM microscopy shows the presence of nanoparticles from 35.15 nm to 59.60 nm. The particles are larger than those visualized by transmission electron microscopy. The particle sizes determined by means of TEM were from 5 nm to 10 nm. The greater particle sizes shown by scanning microscopy are the result of particle agglomeration. Also, figure 5 presents images of synthesized Ag-NPs using the fruit extract of *Ribes nigrum* (a) at a magnification of 100000 X and (b) with selected typical diameters of nanoparticles.

3.5. AFM analysis of Ag-NPs

Using the atomic force microscope (AFM), individual particles and groups of particles can be visualized, and unlike other microscopy techniques, the AFM offers visualization in three dimensions [40]. Also, in this work, synthesized Ag-NPs using the fruit extract of *Ribes nigrum* were characterized by atomic force microscopy. AFM measurements indicate the presence of nanoparticles with typical diameters of about 8 nm. This result is consistent with the measurement of particle diameter using transmission electron microscopy. Figures 6(a)–(d)
presents the AFM images of the synthesized Ag-NPs using the fruit extract of *Ribes nigrum*. Figure 6(a) shows the topography for AFM $5 \mu m \times 5 \mu m$ and figure 6(b) shows the topography for AFM $3 \mu m \times 3 \mu m$. Figure 6(c) presents the topography $1 \mu m \times 1 \mu m$ with the profile of nanoparticles, and figure 6(d) shows the profile for the topography $1 \mu m \times 1 \mu m$.

### 3.6. Antimicrobial activity of Ag-NPs

The antimicrobial activity of the synthesized Ag-NPs using the fruit extract of *Ribes nigrum* towards various microbes was evaluated quantitatively by determining MIC, MBC and MFC. In this study, the efficient antimicrobial activity of synthesized Ag-NPs was found against all tested strains. The results were shown in table 1. The Ag-NPs showed quite similar inhibition activity with MIC of $1 \div 26 \mu g \cdot ml^{-1}$ ($p$-value > 0.05) depending on the tested microorganisms and the species. The synthesized Ag-NPs showed excellently bactericidal activity (the MBC values were only one dilution higher than MIC) and fungicidal activity against dermatophytes *Trichophyton rubrum* ATCC 28188 (MFC was equal to MIC). Moreover, the study revealed that the synthesized Ag-NPs showed excellently antimicrobial activity against both bacteria and fungi, while *Ribes nigrum* fruit water extract, used for preparing Ag-NPs, showed no effect on microbial growth (data not shown). As it is known, smaller

### Table 1. Antimicrobial activity of synthesized Ag-NPs using fruit extract of *Ribes nigrum*.

| Microorganisms                  | Synthesized Ag-NPs | Amikacin | Nystatin |
|--------------------------------|--------------------|----------|----------|
|                                | MIC $\mu g \cdot ml^{-1}$ | MBC/MFC $\mu g \cdot ml^{-1}$ | MIC $\mu g \cdot ml^{-1}$ | MFC $\mu g \cdot ml^{-1}$ |
| *Staphylococcus aureus* ATCC 4163 | 6                  | 6        | 2        | 2        | NT | NT |
| *Pseudomonas aeruginosa* ATCC 6749 | 13                 | 13       | 2        | 2        | NT | NT |
| *Escherichia coli* ATCC 25922   | 1                  | 6        | 2        | 2        | NT | NT |
| *Staphylococcus aureus* K1      | 13                 | 13       | 2        | 2        | NT | NT |
| *Pseudomonas aeruginosa* K1     | 13                 | 26       | 4        | 4        | NT | NT |
| *Candida albicans* ATCC 10231   | 26                 | 26       | NT       | NT       | 8  | 16  |
| *Candida albicans* K1           | 26                 | >26      | NT       | NT       | 16 | 32  |
| *Trichophyton rubrum* ATCC 28188| 6                  | 6        | NT       | NT       | 8  | 32  |
| *Aspergillus niger* ATCC 16404  | 26                 | >26      | NT       | NT       | 32 | 1024 |

Figure 7. Scattergrams show the number of viable cells during various stages of culture. Histograms represent the analysis of cell cycle per unit of time. The marked regions show particular phases of cell cycle, with special emphasis on the percentage of dead cells.

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particles with a larger surface area available for interaction will give more bactericidal effect than larger particles [41]. The smaller the particles, the higher the efficiency of antimicrobial activity. Moreover, Ag-NPs attach to the negatively charged cell surface, alter the physical and chemical properties of the cell membranes and the cell wall [42]. According to AshaRani et al [43], Ag-NPs reduce ATP content of the cell, cause damage to mitochondria and increase production of reactive oxygen species in a dose-dependent manner. Jung et al [44] explained that the mechanism of antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl) groups, but other target sites remain a possibility.

3.7 Evaluation of cell proliferative activity of Ag-NPs

Apart from antimicrobial activity, Ag-NPs have been explored in the field of nano-oncology to recognize their capability for the expansion of cancer treatment [4]. This study also evaluated the cytotoxic activity of Ag-NPs biosynthesized using the fruit extract of Ribes nigrum.

Based on the results of the analysis, it may be concluded that synthesized Ag-NPs using the fruit extract of Ribes nigrum may exercise different kinds of impact on different types of cells. This influence was connected with the concentration of the used factor and it was time-dependent; however, it always resulted in the death of cells. Regardless of cell type, the contact with Ag-NPs caused death to both
malignant endothelial non-small cell lung cancer cells A549 and non-malignant mesodermal lung fibroblasts CCD-39Lu. However, there were observed differences in the mechanisms that led to the cells’ death. This study included the evaluation of cell cycle, which indicated a different sensitivity of malignant and non-malignant cells in particular phases. The analysis of the cell cycle is mainly concerned with the S and G2/M phases, but can also determine the proportion of dead cells. During S-phase, cells replicate DNA, while in G2/M phase cells are prepared to mitosis. The studied cell lines were cultured in the presence of synthesized Ag-NPs, which were added with culture medium in concentrations of 1 µM, 10 µM, and 100 µM. During the period of culture, Ag-NPs caused a dramatic reduction of the number of viable cells, which was accompanied by an increase in the percentage of dead cells, both A549 and CCD-39Lu (figure 7). However, a more detailed analysis indicated differences in the percentage of cells in S and G2/M phases. Under the influence of synthesized Ag-NPs using the fruit extract of Ribes nigrum, A549 and CCD-39Lu cells revealed completely different patterns of activation in S-phase. With the increasing concentration of Ag-NPs, A549 cells were more activated than CCD-39Lu cells, whose activation was decreased, until the complete inhibition in 48 and 72 h. A similar effect was observed in G2/M phase, where CCD-39Lu cells under the influence of 100 µM Ag NPs were inhibited already after 24 h of culture. Lower concentrations of the synthesized Ag-NPs, especially 10 µM caused a similar effect, but it took longer and was not as spectacular. Ag-NPs with the concentration of 100 µM inhibited vital functions of CCD-39Lu cells already during S phase, whereas A549 cells were directed on programmed death pathway in G2/M phase. In the end, the highest concentration of Ag-NPs synthesized using the fruit extract of Ribes nigrum led to the maximum mortality of both A549 and CCD39Lu cells, which was 100 for CCD-39Lu cells (figure 8). Figure 8 presents the percentage of cells in the S and G2/M phases of the cell cycle, and proportion of dead cells of A549 and CCD39-Lu lines cultured in the presence of Ag-NPs assessed at different time points (plots—means; error-bars—SD).

Similar studies were conducted by Mittal et al [45]. They studied the cytotoxic activity of green synthesized Ag-NPs using Potentilla fulgens at different concentrations (10–500 g ml⁻¹) against human lung cancer A549 cell. They showed that synthesized Ag-NPs’ cytotoxicity against human lung cancer A549 cell line was remarkable, with 50% mortality at 100 g ml⁻¹. Moreover, Palaniappan et al [46] studied Ag-NPs biosynthesized using Cymodocea serrulata and their potential cytotoxicity against human lung cancer A549 cells (LD50–100 mg ml⁻¹). Other authors examined the cytotoxicity effect of biologically synthesized Ag-NPs against different cancer cell lines, such as human cervical carcinoma (HeLa) [47], human breast cancer (MCF-7) [48], human Epithelium cells of liver cancer (HepG2) [49] and human acute promyelocytic leukemia (HL-60) cell lines [50].

According to literature, the mechanism involved in Ag-NPs induced cellular toxicity begins with the cellular uptake of inorganic nanoparticles through clathrin-dependent endocytosis and macropinocytosis [51]. Moreover, the hydroxyl radicals released by Ag-NPs cause the damage to cellular components including DNA. The results allow concluding that the A549 and CCD39Lu cells under the influence of the highest concentration of synthesized nanoparticles showed the maximum mortality. Undoubtedly, bioactivator extract of Ribes nigrum fruits contributed to the results. The Ribes nigrum extract itself inhibited different cancer cells, including Caco-2, MCF-7, AGS, MDA-MB-231, and PC-3 [52]. The high content of active compounds also contributed to the achievement of such good results.

4. Conclusion

This study presented the biosynthesis of Ag-NPs using the fruit extract of Ribes nigrum. The prepared Ag-NPs were characterized by UV-visible, SEM, FTIR, TEM and AFM. After 24 h of incubation in the temperature of 25 °C, we obtained spherical particle size of 5–10 nm. The greater particle sizes shown by scanning microscopy are the result of particle agglomeration. The antimicrobial properties of Ag-NPs were tested against bacteria (S. aureus ATCC 4163, P. aeruginosa ATCC 6749, E. coli ATCC 25922), yeast (C. albicans ATCC 10231), filamentous fungi (A. niger ATCC 16404) and dermatophytes (T. rubrum ATCC 28188) as well as clinical isolates of P. aeruginosa K1, S. aureus K1 and C. albicans K1. Based on the obtained results, synthesized Ag-NPs demonstrated the excellent antimicrobial effects. Moreover, biosynthesized Ag-NPs exhibited high efficacy against human lung cancer. Such results provide opportunities in the search of an effective anti-cancer lead.

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