Evaluation of biologically synthesized Au-CuO and CuO-ZnO nanoparticles against glioma cells and microorganisms

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A B S T R A C T
Due to the search for new methods of producing bimetallic nanoparticles, in this work, we have conducted a biological synthesis of Au-CuO and CuO-ZnO nanoparticles using Cnicus benedictus. The synthesized Au-CuO and CuO-ZnO nanoparticles were also analyzed in terms of their antibacterial activity, as well as their influence on cell viability, using two specific cell lines: C6 rat brain glioma (ATCC® CCL-107™) and T98G human glioma (ATCC® CRL-1690®). The studies carried out by means of Atomic Force Microscopy helped to determine the presence Au-CuO nanoparticles whose size was about 13 nm. The size of CuO-ZnO nanoparticles was about 28 nm. The obtained nanoparticles showed cidal activity against glioma cells depending on the concentration of the substance and the time of culture. In the first stage, the nanoparticles limited the ability to divide cells; then, they blocked the cell cycle in the G2 – M phase, and finally led to massive cell death. The antimicrobial activity studies showed that Au-CuO nanoparticles inhibited the growth of microorganisms at lower concentrations than CuO-ZnO nanoparticles, and both kinds of nanoparticles showed excellent cidal properties.

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1. Introduction

The intense development of nanotechnology proves that the current science would not be able to function without this discipline. The production of nanomaterial has a huge potential for a broad spectrum of applications. One of interesting issues in the field of nanotechnology are metal nanoparticles. Due to their sizes, they have different properties than larger particles made of the same material. Bimetallic nanoparticles are composed of two different metals. They attract much attention due to their characteristics that stem from the presence of two single metals and the synergy between them (Sharma et al., 2016; Toshima et al., 1998).

The literature presents numerous examples of obtaining bimetallic nanoparticles by means of physical and chemical methods (Dobrucka & Dlugaszewska, 2018). However, these methods have certain disadvantages; for example, they use costly and toxic reagents or aggressive physical treatments (Loshchinnina et al., 2018). Therefore, more attention has been given to the biological methods of synthesizing nanoparticles with the use of bacteria, fungi or algae. The literature shows few examples of the biological synthesis of bimetallic nanoparticles. One example are the examinations conducted by Ismail et al. (2018), who obtained Cu/Ag and Cu/Ni bimetallic nanoparticles with the use of ginger powder. Kumari et al. (2015) using the fruit juice of pomegranate, obtained Au–Ag bimetallic nanoparticles. In this work, Au-CuO and CuO-ZnO nanoparticles were synthesized with the use of C. benedictus herb water extract. Cnicus benedictus L. (Blessed Thistle or Holy Thistle), the sole species in the genus of Cnicus, is a thistle-like plant from the Asteraceae family (Szabó et al., 2009). C. benedictus usually reaches the height of 30–50 cm but it can also grow up to 70 cm. The flowers are yellow, produced in a dense flower head (capitulum) of 3–4 cm diameter, surrounded by numerous spiny basal bracts (Grigorescu et al., 1986). This plant was chosen due to its rich content of biologically active substances. The herb of C. benedictus is mainly composed of the principal bitter constituents, sesquiterpene lactone glycosides of the germacrane type, among which the chief component is cnicin (0.2–0.7%). The raw material also contains flavonoids, triterpenoids, essential oils, tannins and mineral compounds.
2. Materials and methods

2.1. Preparation of biosynthesized Au-CuO and CuO-ZnO nanoparticles

C. benedictus L. were collected from Lubusz region (Poland). Healthy and fresh C. benedictus L. was washed two times in distilled water, and it was air-dried for 40 days at room temperature. To prepare extract used 4 g powdered C. benedictus L. mixed with deionized water and stirred at 75 °C. Obtained fresh extract was used immediately in all studies after filtration through Whatman’s No. 1 filter paper. In order to obtain the Au-CuO nanoparticles prepared solution of 1 mM of HAuCl4 and 1 mM of CuCl2. Both solutions were mixed with the extract in the 1:1 ratio and stirred at 60 °C for 12 h. In order to obtain the CuO-ZnO nanoparticles prepared solution of 1 mM of HAuCl4 mixed with 1 mM of Zn(NO3)2. The prepared solutions were mixed with the extract, and stirred for 12 h.

2.2. Characterization of biosynthesized Au-CuO and CuO-ZnO nanoparticles

In this work, the UV–VIS absorption spectra of the biosynthesized Au-CuO and CuO-ZnO nanoparticles were obtained from a spectrophotometer Cary E 500 in the range of 300–800 nm using a quartz cell with 10 mm of optical path length. The chemical structure of biosynthesized Au-CuO and CuO-ZnO nanoparticles was investigated using Perkin-Elmer Spectrum 1000. Spectra were recorded in KBr pellets in the range of 380–4000 cm⁻¹. The spectrum was adjusted at a resolution of 4 cm⁻¹. The biosynthesized Au-CuO and CuO-ZnO nanoparticles were characterized using an Atomic Force Microscope (Agilent 5500). The morphology and size of the biosynthesized Au-CuO and CuO-ZnO nanoparticles were characterized using a Transmission Electron Microscope JEOl JEM 1200 EXII, operating at 200 kV and Scanning Electron Microscope (HR SEM) Helios NanoLab 660 (FEI).

2.3. Established cell lines

The influence of the Au-CuO and CuO-ZnO nanoparticles compounds on the viability of cells in vitro was evaluated with use two established cell lines received from American Type Culture Collection (Manassas, VA, USA). The rat glioma brain cell line C6 (ATCC® CCL-17) was cultured in DMEM medium containing 10% fetal bovine serum, 50 U/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B. The human glioblastoma cell line T98G (ATCC® CRL-1690™) was grown in EMEM medium supplemented as mentioned above. Cells were kept at 37 °C in a 5% CO2 atmosphere in the incubator with increased humidity up to 85% and a 30% oxygen concentration inside. Cells were plated in quantity 5 × 10⁵ cells per well and cultured in the plastic 24-Well flat-bottom plates (TC-PLATE 24 well, Greiner).

2.4. The cell viability MTT assay

The rat glioma cell line C6 was incubated with different concentrations of tested compounds for 24 and 48 h. Cell growth inhibition rates were determined with MTT assay. Percentage of cell viability was expressed in relation to untreated control cells. Mean standard deviation (±SD) were calculated using Microsoft Excel software (Microsoft, Redmond, WA, USA) by three independent experiments which were run in duplicates. The half maximal inhibitory concentration (IC50 index) denotes the Au-CuO and CuO-ZnO nanoparticles concentration level that results in a 50% decrease in the cell number compared to non-treated controls. IC50 values were calculated from each time of treatment using the GraphPad software (BIOSOFT, Cambridge, UK). The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay according to manufacturer’s instruction. C6 cells (5 × 10⁴) were cultured overnight in 96-well cell culture plates. First, cells were treated with Au-CuO and CuO-ZnO nanoparticles ranging concentrations (0.5–10 µM). Studied compounds were dissolved in culture media proper for each cell line. After incubation through 24 and 48 h, 10 µL of MTT solution (5 mg-mL) was added to each well 4 h. After that, solubilization solution was applied. Finally, after overnight incubation, the optical density values (OD) were measured at 570 nm using a Multiscan FC spectrophotometer (ThermoScientific, Champaign, IL, USA). As background controls, samples of studied compounds mixed with MTT without test cells were used. The level of absorbance of background control was subtracted from the absorbance of treated and control cells, to diminish of test error. The viability of the control group was set to 100%. The viability of cells cultured under the influence of studied compounds was calculated from the formula:

\[
\text{viability}[^{\%}] = \frac{\text{OD treated cells} - \text{OD control cells}}{\text{OD control cells}} \times 100\%
\]

\text{OD} - \text{mean of the optical density.]

2.5. Flow cytometric analysis of the cell cycle

For tests, C6 and T98c cells were plated in the wells in density 5 × 10⁵ cells-well. Initially, cells were pre-incubated per 24 h and then culture media were removed and replaced with fresh media containing a working solution of test substances. Cell lines were grown through 24, 48 and 72 h in the presence of test nanoparticles in the following concentrations: 100 µM, 10 µM and 1 µM. Cells cultured in media without test substance were used as control samples. Evaluation of the particular phases of the cell cycle was performed using propidium iodide (PI). Measurement of the percentage of cells in the S phase of the cell cycle allows for estimation of the proliferative activity of the studied population. Moreover, for the evaluation of the viability of cells the G2-M cell cycle and dead cells percentage assessment is essential. Distribution of cell cycle phases was investigated through the calculation of the PI mean fluorescence intensity (MFI). When the tests were finished, cells were removed from plates, transferred into tubes and suspended in permeabilization buffer containing 1% of saponin (Sigma). After incubation at 4 °C for 30 min, cells were centrifuged and then kept in PBS buffer containing 10 mg-mL propidium iodide (Sigma) and 100 µg-mL RNase enzyme (Boehringer Mannheim). After another incubation at 4 °C for 30 min, stained samples were subject to acquisition by flow cytometer FACS Canto (Becton Dickinson, USA), and analyzed by FACS Diva software (Becton Dickinson, USA).

2.6. Antimicrobial activity of biosynthesized Au-CuO and CuO-ZnO nanoparticles

Minimal inhibitory concentration (MIC) as well as minimal bactericidal/fungicidal concentration (MBC/MFC) against standard strains of bacteria: Staphylococcus aureus NCTC 4163, Escherichia coli ATCC 25922, Pseudomonas aeruginosa NCTC 6749 and fungal strain Candida albicans ATCC 10231 were determined using broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/). The microbial strains were obtained from the National Collection of Type Cultures (NCTC) and from the American Type Culture Collection (ATCC). The strains were stored by cryopreservation at −80 °C. Prior to use bacterial strains were cultured in brain-heart infusion broth (BHI; OXOID, UK) at 36 °C ± 1 °C for
20 h. and C. albicans cultures were grown in Sabouraud dextrose broth (SDB; OXOID, UK) at 36 °C ± 1 °C for 20 h. The bacteria and C. albicans were harvested by centrifugation (3000 rpm for 15 min), re-suspended in 0.9% NaCl (Avantor Performance Materials Poland S.A.; Poland). Cell suspension were prepared in suitable media – bacteria in Muller-Hinton broth (MHB; OXOID, UK), yeast in SDB to yield the final inoculum concentrations about 1 × 10⁶ CFU/ml. The biosynthesized Au-CuO and CuO-ZnO nanoparticles were two fold diluted to give the final concentration ranging from 80% to 0.15625%. To assess the MICs value 100 μL of each dilution were distributed in 96-well microtiter plates (Kartell, Italy) in quadruple. The cell suspension (100 μL) was then added to each well except the fourth repetition (negative control), to which 100 μL of media was added. The growth control of used microbes were also prepared (contain culture broth and microbial suspension). The plates were incubated at 34 °C for 18 h. The MIC value was defined as the lowest concentration at which no visible growth (no turbidity) was observed. To assess MBC/MFC value, after recording the MIC end point, every well that was demonstrated (no turbidity) was observed. To assess MBC/MFC value, after recording the MIC end point, every well that was demonstrated (no turbidity) was observed. The MIC value was defined as the lowest concentration at which no growth was observed.

3. Results and discussion

3.1. UV VIS studies of biosynthesized Au-CuO and CuO-ZnO nanoparticles

UV–Vis spectroscopy is the most widely used method for characterizing the optical properties and electronic structure of nanoparticles as the absorption bands are related to the diameter of metal nanoparticles (Philip, 2008). According to the literature (Feldheim and Foss, 2002) light wavelengths in the 300–800 nm are used for characterizing various metal nanoparticles. Also, the optical absorption spectra of the biosynthesized Au-CuO and CuO-ZnO nanoparticles were recorded at the range of 300–800 nm. In this study, the UV-absorption spectrum of the synthesized Au-CuO and CuO-ZnO nanoparticles was monitored after 12 h of stirring and heating. As regards the Au-CuO nanoparticles spectrum, an intensive peak was observed at 350 nm and at 650 nm. The CuO-ZnO nanoparticles spectrum showed similar results, with a strong peak at 350 nm. Fig. 1 presents the UV-visible spectra of Au-CuO and CuO-ZnO nanoparticles biosynthesized with the use of C. benedicti herba.

3.2. FTIR of biosynthesized Au-CuO and CuO-ZnO nanoparticles

Fig. 2 presents the FTIR spectra of (A) Au-CuO and (B) CuO-ZnO nanoparticles biosynthesized with the use of C. benedicti herba. Figure A presents the strong peaks which were observed at 3305 cm⁻¹, 2124 cm⁻¹, 1634 cm⁻¹, 413 cm⁻¹, 398 cm⁻¹ and 387 cm⁻¹. Figure B shows the strong peaks which were observed at 3308 cm⁻¹, 2124 cm⁻¹, 1634 cm⁻¹, 390 cm⁻¹, 396 cm⁻¹, 412 cm⁻¹ and 431 cm⁻¹. The strong peaks at 3305 cm⁻¹ and 3308 cm⁻¹ stretching in FTIR correspond to the OH group, which confirms the presence of phenolic compounds in C. benedicti herba extract. The intensive peaks at 2124 cm⁻¹ may indicate the presence of the alkene group. The absorption band at 1634 cm⁻¹ can be assigned to the N-H bending vibration of amine or amide groups. The bands at 390 cm⁻¹ and 396 cm⁻¹ are attributed to the deformation vibration of C-C in polymer chains. The absorption bands at 387 cm⁻¹ (Figure A) and 412 cm⁻¹ (Fig. 1) indicated the formation of metal-biomolecules present in the extract.

Fourier transform infrared spectrum confirmed the presence of biologically active compounds in C. benedicti herba extract. It has been assumed that bioactive compounds act as the reducing and capping agents for Au-CuO and CuO-ZnO nanoparticles. C. benedicti herba contains sesquiterpene lactones (cnicin, salotoninolide, artemisiifolin), lignan lactones (arc tigenin, nortracheloside, 2-acetyl trachelogenin), essential oils (p-cymene, citronellol, cuminal, fenchon, cinnamaldehyde and benzaldehyde) (Van Wyk et al., 2004; Chevalier et al., 2000; Bryan et al., 2008; Vanhaelen-Fastre, 1973) triterpenoids (α-amyrinone, α-amyrin, acetate, multiflorenol, multiflorenol acetate and oleanolic acid, phytosterols, flavonoids and mineral salts. The presence of flavonoids is very significant for the formation of Au-CuO and CuO-ZnO nanoparticles. C. benedicti contains such flavonoids as apigenin-7-O-glucoside, luteolin and astragalin (Uljeben and Berkman, 1977). The antioxidant activity of flavonoid compounds is associated with the ring-shaped structure of the particle, which
contains conjugated double bonds, as well as with the presence of various functional groups in the rings (Panche et al., 2016). The intensity of their antioxidant activity depends on the number and position of hydroxyl groups. Compounds with a greater number of hydroxyl groups have stronger antioxidant properties. As antioxidants, they can act by means of direct reactions with free radicals, as well as by scavenging free radicals, intensifying the disproportionation of free radicals to much less reactive compounds, chelating pro-oxidant metals, and inhibiting or strengthening the activity of numerous enzymes. Fig. 3 presents the chemical structure of (A) arctigenin, (B) salonitenolid, (C) knicin and (D) trachelogenin.

3.3. AFM studies of biosynthesized Au-CuO and CuO-ZnO nanoparticles

AFM (atomic force microscopy) was used to determine the size of the Au-CuO and CuO-ZnO nanoparticles biosynthesized with the use of C. benedicti. Figure shows the AFM image of biosynthesized Au-CuO nanoparticles with (A) the topography $10 \mu m \times 10 \mu m$, (B) the topography $3 \mu m \times 3 \mu m$, (C) the topography $1.5 \mu m \times 1.5 \mu m$ with the profile. Fig. 4 presents the AFM image of biosynthesized CuO-ZnO nanoparticles with (D) the topography $10 \mu m \times 10 \mu m$, (E) the topography $3 \mu m \times 3 \mu m$ and (F) the topography $1.5 \mu m \times 1.5 \mu m$ with the profile. On the basis of the obtained results, it was determined that the size of the nanoparticles was about 13 nm (Au-CuO) and about 28 nm (CuO-ZnO).

3.4. TEM and SEM of biosynthesized Au-CuO and CuO-ZnO nanoparticles

The size and morphology of the Au-CuO and CuO-ZnO nanoparticles prepared with the use of C. benedicti were analyzed by transmission electron microscopy and scanning electron microscopy. According to Schaffer et al. (2009), transmission electron microscopy is an extremely useful technique to obtain the direct information concerning mean particle size and the shape of
nanoparticles. Fig. 5 shows TEM images of (A) biosynthesized Au-CuO nanoparticles (200,000× magnification), (B) CuO-ZnO nanoparticles (100,000× magnification) and SEM images of (C) Au-CuO nanoparticles (2000× magnification), (D) Au-CuO nanoparticles (5000× magnification) (D) CuO-ZnO nanoparticles (500× magnification) and (E) CuO-ZnO nanoparticles.

Fig. 4. AFM image of biosynthesized of Au-CuO nanoparticles with (A) the topography 10 μm × 10 μm, (B) the topography 3 μm × 3 μm, (C) the topography 1.5 μm × 1.5 μm with the profile, and CuO-ZnO nanoparticles using of C. benedicti herba (D) the topography 10 μm × 10 μm, (E), the topography 3 μm × 3 μm and (F) the topography 1.5 μm × 1.5 μm with the profile.
Spherical Au-CuO and CuO-ZnO nanoparticles were observed. At some places, the particles were aggregated. It was especially visible in the case of CuO-ZnO nanoparticles, mainly as a result of the high surface energy of ZnO nanoparticles, which is usually generated when synthesis is conducted in an aqueous environment (Alessio et al., 2008). Another reason may be the densification that results in narrow spaces between particles (Ryu et al., 2002). Scanning electron microscopy confirmed that the size of Au-CuO nanoparticles was about 20 nm, and the size of CuO-ZnO nanoparticles was about 30 nm. Those results are very similar to those obtained by means of atomic force microscopy.

3.5. The effect of biosynthesized Au-CuO and CuO-ZnO nanoparticles on C6 cells viability

We evaluated the effect of Au-CuO and CuO-ZnO nanoparticles on the viability of rat glioma C6 cells using the MTT assay. After 24 h and 48 h treatment, the IC50 value was calculated (Fig. 6). The cell viability was markedly decreased when exposed to
Fig. 6. The cytotoxicity of biosynthesized Au–CuO and CuO-ZnO nanoparticles in relation to C6 cells.

|           | Au-CuO-NP            | CuO-ZnO-NP            |
|-----------|----------------------|-----------------------|
| 24h I_{50} (μM) ± SD | 0.90751 ± 2.4211      | 4.91128 ± 2.6430      |
| 48h I_{50} (μM) ± SD | 0.53663 ± 2.0396      | 3.37202 ± 1.4861      |

* values are means ± SD [n=6]

Fig. 7. Histograms showing the effect of biosynthesized CuO-ZnO nanoparticles compounds at different concentrations (control: 0 μM, 1 μM, 10 μM and 100 μM) on rat glioma C6 cells and human glioblastoma T98G cells at 48 h of culture.
Au-CuO nanoparticles at concentrations from 0.5 μM to 10 μM. 50% decrease in viable cells was noted at 0.9075 μM and 0.5366 μM for 24 h and 48 h incubation with Au-CuO nanoparticles, respectively. Weaker inhibition of cellular proliferation was observed after treatment with CuO-ZnO nanoparticles. IC50 for C6 cells was 4.9113 μM and 3.3720 μM for 24 h and 48 h incubation, respectively. The presented data showed that Au-CuO and CuO-ZnO nanoparticles inhibited cell viability in a dose- and time-dependent manner. Notably, Au-CuO nanoparticles displayed approximately five times higher cytotoxicity on the viability of glioma cell line, than the second tested compound. Presented differences in activity may occur due to a different substituent.

3.6. The effect of biosynthesized Au-CuO and CuO-ZnO nanoparticles on the cell cycle phases

Results of flow cytometric analysis indicated that studied nanoparticles affected the tested glioma and glioblastoma cell lines...
in a similar manner. Contact with of Au-CuO and CuO-ZnO nanoparticles molecules led cells to death. However, their impact was related to the concentration of used substance and was time-dependent. Au-CuO nanoparticles as compared to CuO-ZnO nanoparticles have a stronger effect on cell death, both C6 and T98G. The study revealed also differences in the sensitivity to test substances between the cell lines used. For both of Au-CuO and CuO-ZnO nanoparticles, rat C6 cell lines showed a much higher susceptibility to the cytotoxic effect than human glioblastoma cells T98G (Fig. 7).

Depending on the concentration of the substance and the time of culture, the nanoparticles, in the first stage, limited the ability to divide cells, then blocked the cell cycle in the G2 – M phase, and finally led to massive cell death. In the most obvious way, this effect was visible for C6 cells. In most cases, the most pronounced effect was observed with both substances at the highest concentration, i.e. 100 μM. However, in the case of the C6 line, a very strong effect was observed with Au-CuO nanoparticle substances already at the concentration of 10 μM (Figs. 8 and 9).

The observed excellent anticancer properties of the examined preparations stem not only from the presence of nanoparticles, but also from the use of Cnici benedicti herba extract. The sesquiterpene lactones present in Cnici benedicti herba (oxidized sesquiterpene derivatives) are organic compounds that originate from terpenes.

Fig. 9. Percentage of C6 and T98G cells cultured in the presence of the biosynthesized CuO-ZnO nanoparticles compounds in the S and G2-M phases of a cell cycle and a proportion of a dead cells, measured in various periods of time (plots – means; error-bars SD). Values statistically different at p < 0.05 (△ = 100 μM; ± 10 μM) in Kruskal-Wallis test.
Gram-positive and gram-negative bacteria, as well as yeast. The activity against a broad spectrum of microorganisms, both antibacterial and fungicidal concentration (MBC/MFC) against standard strains of bacteria: Staphylococcus aureus NCTC 4163, Escherichia coli ATCC 25922, Pseudomonas aeruginosa NCTC 6749 and fungal strain Candida albicans ATCC 10231. The results of the antimicrobial activity assay of biosynthesized Au-CuO and CuO-ZnO nanoparticles showed that the obtained Au-CuO nanoparticles inhibited the growth of microorganisms at lower concentrations than CuO-ZnO nanoparticles. These screening tests indicate that biosynthesized Au-CuO and CuO-ZnO nanoparticles are potential candidates for antimicrobial agents. Based on the obtained results, it may be concluded that the synthesis and use of bimetallic nanoparticles have opened new avenues in different fields, and their application as antimicrobial agents may be very useful during the post-antimicrobial resistant era (Syed et al., 2018).

4. Conclusion

Metal nanoparticles, as one of the solutions proposed by nanotechnology, have come into focus due to their wide and highly interesting properties. The growing interest in bimetallic nanoparticles has created the need for finding new methods of their synthesis. One example is biological synthesis, which has gained considerable attention in the recent years due to its potential applications in various fields of science. In this paper, we have reported on the biologically synthesized Au-CuO and CuO-ZnO nanoparticles produced with the use of C. benedicti. The assessment of the optical properties, size and shape of the bimetallic nanoparticles was carried out by means of such methods as: UV–vis, FTIR, SEM, TEM and AFM. We have observed spherical Au-CuO and CuO-ZnO nanoparticles whose size was about 13 nm and 28 nm respectively. FTIR confirmed the presence of biologically active compounds in C. benedicti herba extract. The obtained Au-CuO and Cu-ZnO nanoparticles showed cidal activity against glioma cells, which depended on the concentration of the substance and the time of culture. In the first stage, the nanoparticles limited the ability to divide cells, then blocked the cell cycle in the G2 – M phase, and finally led to massive cell death. In the case of the C6 line, a very strong effect was observed with Au-CuO nanoparticles substances already at the concentration of 10 μM. The studies of the antimicrobial activity of biosynthesized Au-CuO and CuO-ZnO nanoparticles showed that the obtained Au-CuO nanoparticles inhibited microbial growth at lower concentrations than CuO-ZnO nanoparticles.

Conflict of interest

The authors declare that they have no conflict of interest.
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