



Anti-Leukemia Activity of Au/CuO/ZnO Nanoparticles Synthesized using *Verbena officinalis* Extract

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Abstract

As biological synthesis has become an alternative to chemical and physical methods for synthesizing nanoparticles, this work describes the synthesis of Au/CuO/ZnO nanoparticles using *Verbena officinalis* extract. The synthesized Au/CuO/ZnO nanoparticles were characterized using Ultraviolet–Visible, Fourier Transform–Infrared, Transmission Electron Microscopy and Atomic Force Microscopy. The influence of Au/CuO/ZnO nanoparticles on cell viability was evaluated in vitro, using the established cell line – Jurkat (ATCC® TIB-152™). The Annexin V binding test confirmed the previous results of the MTT assay, which indicate that the studied complex of Au/CuO/ZnO nanoparticles has a strong cytotoxic effect on the Jurkat cell line. The type of death and the effectiveness of cell elimination depended both on the concentration of the complex and the duration of culture.

Keywords Biosynthesis · *Verbena officinalis* · Leukemia

1 Introduction

Recently, metal nanoparticles have been the subject of extensive studies due to the usefulness of their biological properties, benign nature and great characteristics [1]. Changes in the physical and chemical properties of this class of nanomaterials can heavily influence their applications [2]. Many scientists have focused on bimetallic and trimetallic nanoparticles, whose chemical and physical properties are different from the properties of individual metals because of combinations and interactions between the individual

components of such nanoparticles. Nanoparticle combinations give rise to the search for and design of new materials (nanomaterials) with unique characteristics, which attracts the attention of researchers working in various engineering and academic fields [3].

There are various methods for synthesizing trimetallic nanoparticles, including microwave, co-precipitation, hydrothermal, selective catalytic reduction and micro emulsion etc. The microwave dielectric heating offers considerable advantages as it makes it possible to tailor the structural aspect by controlling the conditions of reactions [4]. Trimetallic nanoparticles are also obtained from biological synthesis [5]. Biological substrates are used in the preparation and development of bio-medically useful inorganic nanoparticles due to their non-toxic properties and environmentally friendly nature [6]. Moreover, biological synthesis is straight forward and uncomplicated, while the bioactivity of biosynthesized nanoparticles is enhanced by plant phytonutrients. Recently, with the increasing awareness of risks associated with conventional methods and the negative impact of chemical methods on the environment, green synthesis has become more significant. Even though conventional chemical methods make it possible to easily control, to a certain extent, the shape of synthesized nanoparticles, they have two major drawbacks: the cost and the application of toxic chemicals [1].

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Plants are considered as natural capping agents for nanoparticle synthesis and microorganisms are considered as nanofactories. The intracellular and extracellular metal nanoparticles are synthesized from bacteria, fungi, algae and actinomyces. Besides methods of extracellular synthesis of silver nanoparticles, more attention is paid towards the intracellular method. Due to the presence of biologically active compounds, trimetallic Au/CuO/ZnO nanoparticles were synthesized using *Verbena officinalis* water extract. The use of biological synthesis using *V. officinalis* water extract to obtain trimetallic nanoparticles has not been reported in the literature. The novelty is the biological synthesis of trimetallic nanoparticles using this type of plant.

V. officinalis is the main species belonging to the *Verbena* genus of the Verbenaceae family (Verbenaceae subfamily). The whole Verbenaceae family consists of about 1100 species. It includes trees, bushes and herbaceous plants. *V. officinalis* is naturally distributed throughout the world: in Europe, both Americas, North and Central Africa, Asia and Australia. The species grows mainly in the temperate climate zone. In Europe, it is commonly found in the Mediterranean. The main groups of secondary metabolites that determine the biological activity profiles of the raw material are iridoid glycosides, including verbenalin (verbenalioside), aucubin (verbenine) and hastatoside, as well as phenylpropanoid glycosides (caffeic acid derivatives), including verbascoside (acteoside), isoverbascoside (isoacteoside), and eukovoside [7].

2 Materials and Methods

2.1 Materials

Chemicals used in this work were purchased from Sigma–Aldrich (Poland).

2.2 Synthesis of Au/CuO/ZnO Nanoparticles using *Verbena officinalis* Extract

Au/CuO/ZnO nanoparticles were synthesized using *V. officinalis* water extract. The first stage of the study started with preparing the extract. The extract was made from 5 g of finely powdered *V. officinalis*, to which 100 ml of double distilled water were added. The prepared solution was subjected to heating and vigorous stirring for 55 min at 90 °C. During that time, three separate solutions were prepared: 5 mM H₂AuCl₄, 5 mM CuCl₂, and 5 mM Zn(NO₃)₂. The obtained fresh extract was filtered through Whatman's No. 1 filter paper and combined with the prepared solutions at a 1:1:1:1 ratio. Then, the solution was stirred for 24 h at 55 °C. It is important to maintain a constant temperature and a stirring speed.

2.3 Characterization of Au/CuO/ZnO Nanoparticles using *Verbena officinalis* Extract

The maximum absorbance of the sample was measured by UV–Visible spectrophotometry. Ultraviolet and visible absorption spectroscopy (spectrophotometer Cary E 500) in the range of 300–600 nm were used to analyze the optical properties. The characterization of Au/CuO/ZnO nanoparticles involved Fourier Transform Infrared Spectroscopy analysis with Perkin Elmer Spectrum 1000 (USA), in attenuated total reflection mode and using spectral range of 4000 – 380 cm⁻¹, with a resolution of 4 cm⁻¹. FTIR analysis was used to determine the binding properties of Au/CuO/ZnO nanoparticles synthesized using *V. officinalis* extract. Fourier transform infrared spectroscopy (FTIR) was used to characterize Au/CuO/ZnO nanoparticles synthesized using *V. officinalis* extract. The shape, size and microstructures of Au/CuO/ZnO nanoparticles synthesized using *V. officinalis* extract were determined by means of a Transmission electron microscope JEOL JEM 1200 EXII operating at 80 kV. The study was conducted using the atomic force microscope INTEGRA SPECTRA SOLAR of NT-MDT brand, and measurement tips dedicated for NSGO1 high-resolution measurements, in the tapping mode. The resonance frequency of the tips ranged from 87 to 230 kHz. The force constant ranged from 1.45 to 15.1 N/m. The scanning area was 10 μm x 10 μm, with 1000 × 1000 scanning points. The picture of Au/CuO/ZnO nanoparticles synthesized using *V. officinalis* extract was made by means of Scanning electron microscopy (SU3500), Hitachi.

2.4 Evaluation of Cytotoxic Activity of Au/CuO/ZnO Nanoparticles using *Verbena officinalis* Extract

The impact of Au/CuO/ZnO nanoparticles on cell viability was evaluated in vitro, using the established cell line—Jurkat (ATCC® TIB-152™) obtained from American Type Culture Collection (Manassas, VA, USA). Acute T Cell Leukemia cell line—Jurkat was cultured in the RPMI-1640 medium with 10% of fetal bovine serum (FBS), 50 U/mL of penicillin, 100 μg/mL of streptomycin, and 25 μg/mL of Amphotericin B. Cells were distributed in the amount of 5 × 10⁵ cells per well, and cultured in an incubator on 24-well plastic plates with flat bottom (TC-PLATE 24 well, Greiner) at 37 °C, 5% CO₂ atmosphere with increased humidity. Au/CuO/ZnO nanoparticles were added at three different concentrations: 1 μM, 10 μM, and 100 μM. Samples without the addition of Au/CuO/ZnO nanoparticles served as controls. All the tested samples, both Jurkat cells, and PBMC were cultured for the period of 24, 48 and 72 h.

2.5 PBMC Separation

Peripheral blood samples from voluntary donors were collected to test tubes with heparin. Then, blood was mixed at a 1:1 ratio with sterile Phosphate-Buffered Saline (PBS), and placed on the Histopaque®-1077 (Sigma-Aldrich) solution. Histopaque®-1077 is a polysucrose and sodium diatrizoate solution with the density of 1.077 g/mL, which enables the separation of viable lymphocytes and other mononuclear cells from the other components of whole peripheral blood (thrombocytes, erythrocytes, granulocytes). After 20 min of centrifugation at 1500 rpm in RT, the interphase containing peripheral blood mononuclear cells (PBMC) was collected and transferred onto 24-well culture plates. Then, PBMC cells were cultured in RPMI 1640 media with 10% of FBS and 1% of antibiotic/antimycotic mixture, as described above for the Jurkat cell line. Following the culture in the presence of the studied Au/CuO/ZnO nanoparticles, PBMC were subjected to cell cycle assessment.

2.6 MTT Test (Cell Viability Assay)

The Jurkat cell line was incubated with different concentrations of the tested substance for 24 and 48 h. The inhibition of cell growth caused by nanoparticles was determined by MTT assay. The viability percentage of Jurkat cells was specified in reference to control cells cultured without the addition of the studied nanoparticles. The mean standard deviation (\pm SD) was calculated with the use of Microsoft Excel software (Microsoft, Redmond, WA, USA) in three independent experiments repeated twice. Half-maximal inhibitory concentration (IC₅₀) means the concentration of Au/CuO/ZnO nanoparticles that causes a 50% reduction in the number of cells compared to non-stimulated controls. IC₅₀ values were calculated for each time point of culture using CalcuSyn software (BIOSOFT, Cambridge, United Kingdom). Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in accordance with the manufacturer's instructions. Jurkat cells (5×10^3) were cultured with the addition of Au/CuO/ZnO nanoparticles at the following concentrations: 0.1; 0.5; 1; 2; 4; 6; 8; 10; 50 and 100 μ mol. The studied cells were suspended in the culture medium in which the cell line was cultured. After 24, 48 and 72 h of incubation, 10 μ l of the MTT solution (5 mg/mL) were added to each well for 4 h. In order to release formazan crystals from the cells, 100 μ l of a solubilizing solution were added to the samples, which were then incubated overnight. Then, optical density (OD) values of the samples were measured with a spectrophotometer Multiscan FC (ThermoScientific, Champaign, IL, USA) in the wavelength of 570 nm. Samples of the studied compounds mixed with MTT without the test cells were used as negative controls. In order to reduce test error, the

background control absorbance level was subtracted from the absorbance of tested and control cells. The viability of the control group was determined as 100%. Test results were given as Relative Viability of Cells (RVC), which is defined as the ratio between the absorbance value of cells cultured in the presence of the tested nanoparticles and the absorbance value of control samples. The RVC was calculated on the basis of the formula described below, where (a) stands for the absorbance of the tested sample; (b) stands for the absorbance of the blank control sample (pure medium without cells); and (c) stands for the absorbance of control cells without the addition of Au/CuO/ZnO.

$$\text{RVC (\%)} = [(a - b)/(c - b)] \times 100$$

The IC₅₀ index was calculated as well. The IC₅₀ value shows the concentration of tested substances which is needed to inhibit, *in vitro*, the biological activity of cells by 50%.

2.7 Evaluation of Apoptosis with Annexin V

The impact of Au/CuO/ZnO nanoparticles on the viability of the Jurkat cell line was also evaluated in terms of the initiation of apoptosis or necrosis. The evaluation of apoptotic or necrotic death was carried out using a commercially available FITC Annexin V apoptosis detection kit (BD Pharmingen), in accordance with the manufacturer's protocol. Analogously to the MTT assay, cells were cultured in the presence of 1 μ M, 10 μ M, and 100 μ M of Au/CuO/ZnO nanoparticles for 24 and 48 h. The test was performed as follows: first, cells were suspended in 100 μ l of Annexin buffer (1 \times) in the amount of 1×10^5 cells. Next, 5 μ l of propidium iodide (PI) and 5 μ l of Annexin V conjugated with fluorescein (FITC) were added to the samples. After 15 min of incubation in the dark, 400 μ l of Annexin buffer (1 \times) were added to each test tube. Stained samples were acquired with FACS Canto flow cytometer (Becton Dickinson), and results were analyzed with FACS Diva software (Becton Dickinson). Cells were defined as early apoptotic, late apoptotic or necrotic based on the proportion between FITC and/or PI fluorescence.

2.8 Flow Cytometry of Cell Cycle

In order to evaluate the cell cycle of the Jurkat cell line, cells were placed in the wells in the amount of 5×10^5 cells per well. The cell line was cultured for 24, 48 and 72 h in the presence of the tested nanoparticles at the concentration of 1, 10 and 100 μ mol. Cells cultured in the medium without the tested substance were used as control samples. The cell cycle of PBMC cells exposed to the studied nanoparticles was evaluated as well. The respective cell cycle phases were assessed using propidium iodide (PI). The measurement of

the percentage of cells in the S phase of the cell cycle makes it possible to estimate the proliferative activity of the population. Moreover, the evaluation of the cell cycle makes it possible to determine the percentage of dead cells and cells in the G2/M phase. The distribution of cell cycle phases was examined by calculating the mean fluorescence intensity (MFI) of PI. After the tests, cells were transferred from plates to test tubes and suspended in the permeabilization buffer—BD Perm/Wash™ (BD Biosciences). After incubation for 30 min at 4 °C, cells were centrifuged and suspended in a PBS buffer with 10 mg/mL of propidium iodide (Sigma) and 100 µg/mL of RNase enzyme (Boehringer Mannheim). After further incubation for 30 min at 4 °C, stained samples were acquired with FACS Canto flow cytometer (Becton Dickinson, USA), and analyzed with FACS Diva Software (Becton Dickinson, USA).

3 Results and Discussion

3.1 UV–VIS Studies of Au/CuO/ZnO Nanoparticles

In the UV–Visible study, UV–Vis spectroscopy was used to monitor the formation rate of nanoparticles. In this study, the absorbance of the solution was measured in a wavelength range of 200–800 nm. Figure 1 presents the UV–Visible spectra of trimetallic nanoparticles after reaction at 55 °C for 12 h, 18 and 24 h and *Verbena officinalis* extract. After 24 h, there were clearly visible peaks at 620 nm and 300 nm, which indicated the emergence of nanoforms of the salts used in the study. The UV absorption spectrum determined the absorption band below 400 nm is characteristic of ZnO nanoparticles [8]. The studies of Wahaba et al. [9] where absorption peak at 364 nm which indicates the presence of

pure ZnO nanoparticles. The absorption peak of ZnO nanoparticles below 400 nm obtained in the range of 373 nm [10], 375 nm [11] and 370 nm respectively [12]. Moreover, a change in color visually confirmed the formation of trimetallic nanoparticles. After the extract was mixed with metal nanoparticle precursors, the solution rapidly changed its color from light beige to dark beige (Fig. 2). Such a visible change of color confirms the reduction of metals to the nanoform. The similar studies conducted Paul et al. [8] and Suwannarat et al. [13], which synthesized Ag/Au/Pd nanoparticles for reduction of 4-nitrophenol.

3.2 Fourier Transform Infrared Spectroscopy (FTIR) Studies of Au/CuO/ZnO Nanoparticles

The results of the FTIR analysis of Au/CuO/ZnO nanoparticles synthesized using *Verbena officinalis* extract are presented in Fig. 3. The FTIR spectra of the extract showed major absorption bands at 3273 cm⁻¹, 2107 cm⁻¹, 1635 cm⁻¹, 418 cm⁻¹, 385 cm⁻¹ and 383 cm⁻¹. The peak at 3273 cm⁻¹ mainly corresponds to symmetrical stretching of H₂O. The peak which appeared at 2107 cm⁻¹ may indicate the presence of an alkynes group. The most intense band at 1635 cm⁻¹ represents C=O vibrations typical of flavonoid structure (but also would have contribution of the scissoring deformation of H₂O). The peaks at 418 cm⁻¹, 385 cm⁻¹ and 383 cm⁻¹ are related to anti-symmetric stretching mode of the surface bridging oxide formed by condensation of the adjacent surface hydroxyl groups. The results confirm that *V. officinalis* contains biologically active compounds.

According to the literature, *V. officinalis* herb extracts contain numerous flavonoids, such as kaempferol, luteolin, apigenin, scutellarein and pedalitin. The biological activity of *V. officinalis* is highly influenced by the presence

Fig. 1 UV–Visible spectra of Au/CuO/ZnO nanoparticles and *V. officinalis* extract

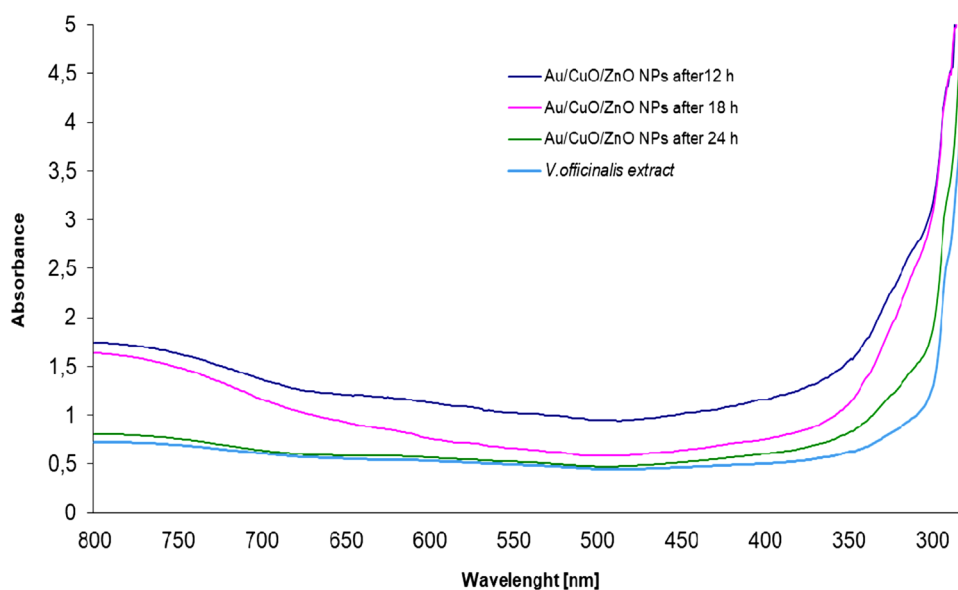
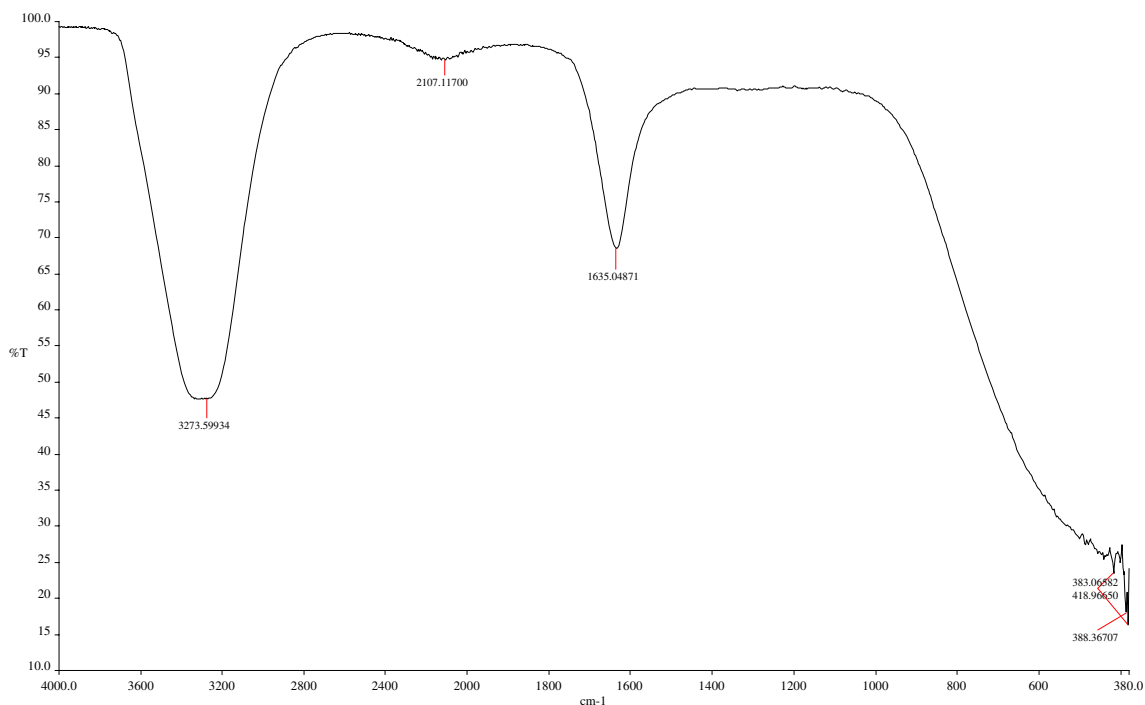
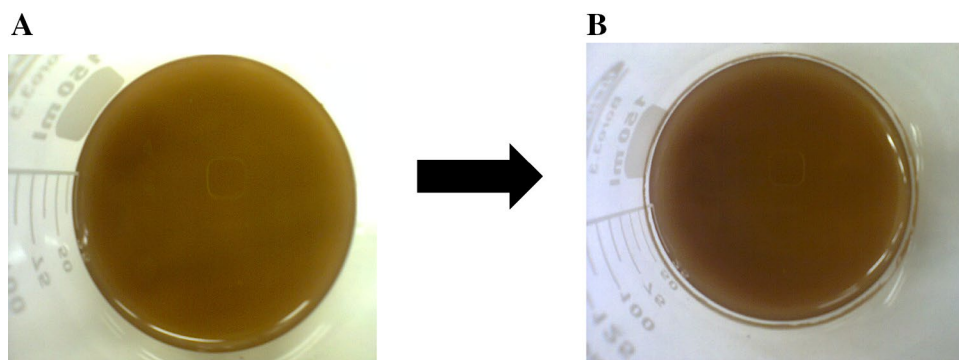


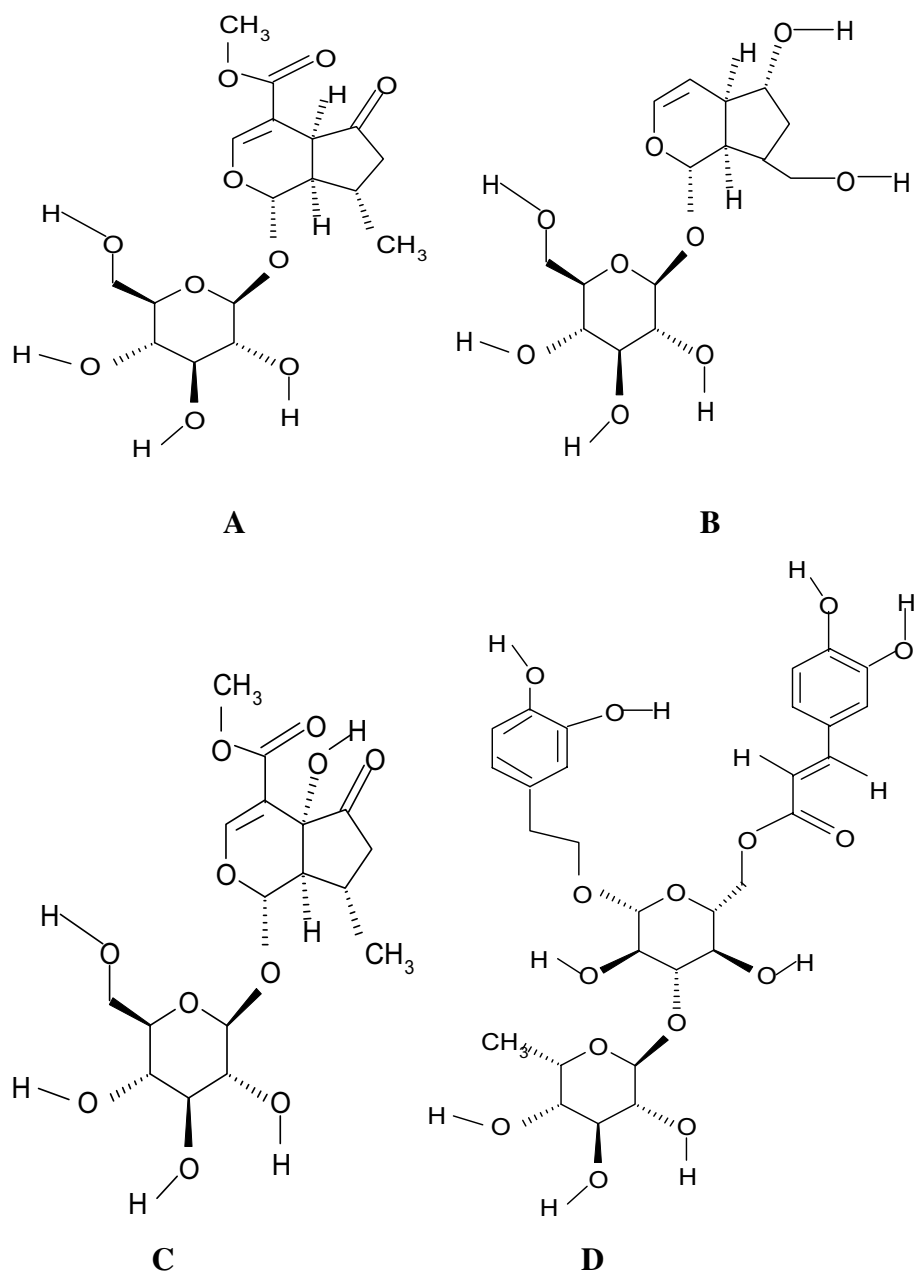
Fig. 2 Solution before (a) and after reaction at 55 °C (b)**Fig. 3** FTIR spectra of the Au/CuO/ZnO nanoparticles synthesized using *V.officinalis* extract

of phenolic acids: chlorogenic, ferulic, protocatechuic, rosmarinic acid and dicaffeoylquinic acid derivatives [7]. Phenolic acids have strong antioxidant properties, which, as shown by scientists, are chemical structure-dependent. The antioxidant activity is related to the number of hydroxyl groups in the particle as well as their degree of esterification. If only one hydroxyl group is present in the compound, its antioxidant properties are strengthened by one or two methoxy groups in the ring. The antioxidant properties of phenolic acids are further stabilized by an alkyl group or a methoxy group, i.e. a group that contains electron donors, introduced in the ortho– position.

The herb of *V. officinalis* contains essential oil with about 40 compounds, the majority of which are

monoterpenes (such as limonene, citral, cineole, carvone) [14]. Other groups of terpene compounds characteristic of the oil are diterpenes (carnosol, carnosic acid, rosmanol, isorosmanol) and triterpenes (ursolic acid and its derivatives). The herb of *V. officinalis* also contains carbohydrates such as d-Galacturonic acid, arabinose, galactose, rhamnose, xylose, mannose or glucose. Moreover, the main constituents of *V. officinalis* include iridoid glycosides: verbenalin (verbenalloside), aucubin (verbenin) and hastatoside, and phenylpropanoid glycosides: verbascoside (acteoside, eucovoside) and isoverbascoside (isoacteoside) [15]. Figure 4 shows the chemical structure of active compounds in *V. officinalis*: (A) verbenalin, (B) aucubin, (C) hastatoside, (D) isoverbascoside.

Fig. 4 The chemical structure of active compounds in *V. officinalis* **a** verbenalin, **b** aucubin, **c** hastatoside, **d** isoverbasoside



3.3 Atomic Force Microscopy (AFM) Studies of Au/CuO/ZnO Nanoparticles

The size of the synthesized Au/CuO/ZnO nanoparticles was determined by means of Atomic force microscopy. The obtained nanoparticles were spherical in shape and 35 nm in size. There were also observed single agglomerates of particles of about 50 nm. AFM microscopes can be used to carry out topographic measurements and to determine certain mechanical properties of the samples, such as elasticity, adhesion force and friction. Figure 5 presents the AFM image of the Au/CuO/ZnO nanoparticles with

the topography of (A) (C) $10\ \mu\text{m} \times 10\ \mu\text{m}$, (B) $3\ \mu\text{m} \times 3\ \mu\text{m}$, (D) $3\ \mu\text{m} \times 3\ \mu\text{m}$ with the profile.

3.4 Transmission Electron Microscope Analysis (TEM) Studies of Au/CuO/ZnO Nanoparticles

TEM images were used to characterize the structure of Au/CuO/ZnO nanoparticles. As indicated by their morphologies, the majority of the synthesized trimetallic nanoparticles were spherical in shape. Figure 6 shows images of the synthesized Au/CuO/ZnO nanoparticles with the following scale bars: (A), (B) 100 nm; and (C), (D) 200 nm.

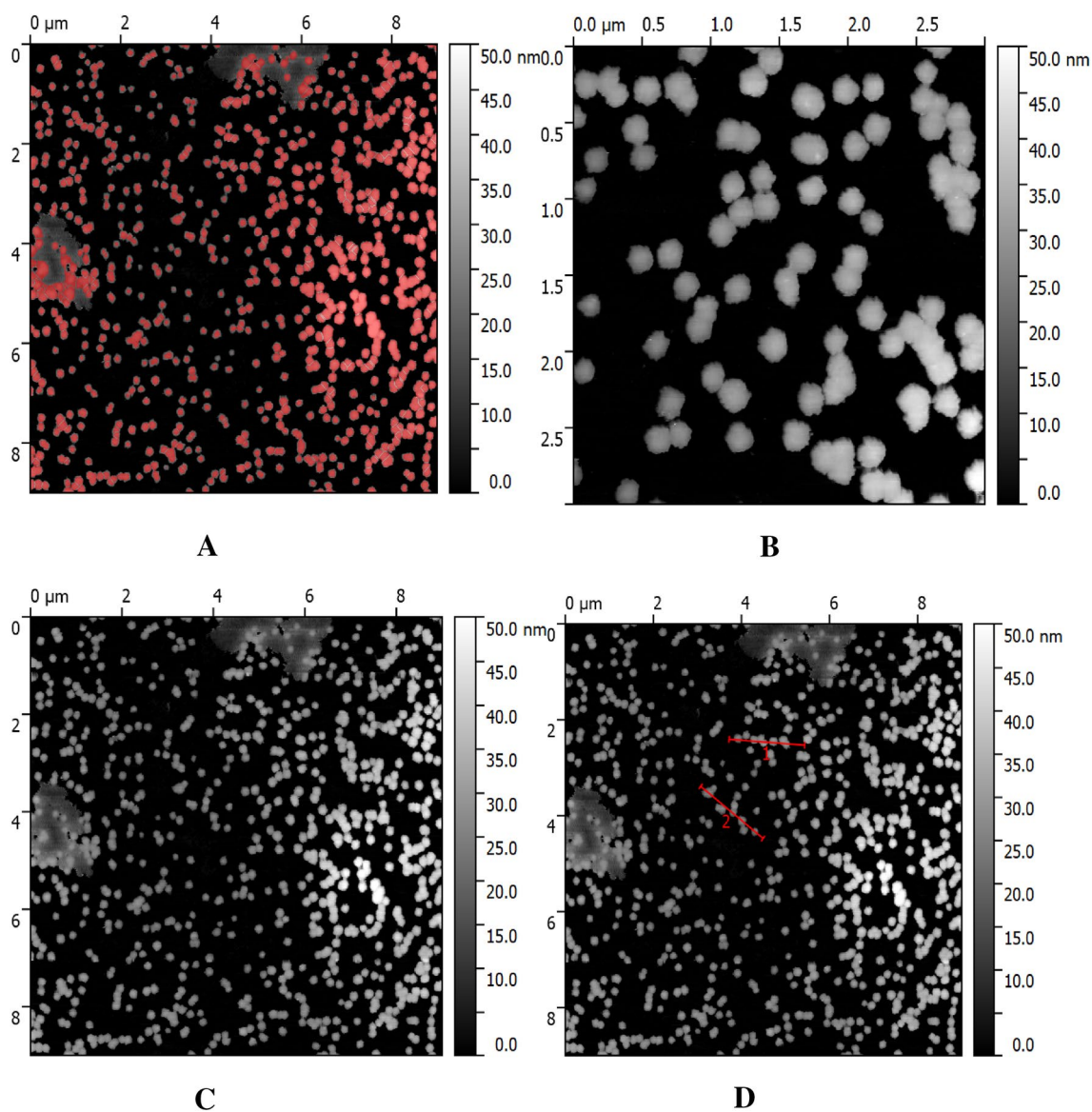


Fig. 5 AFM image of the Au/CuO/ZnO nanoparticles synthesized using *V.officinalis* extract

3.5 Cytotoxic Activity of Au/CuO/ZnO Nanoparticles Synthesized using *Verbena officinalis* Extract

3.5.1 MTT Assay

Leukemia is a heterogeneous group of diseases that involve malignant cloning and proliferation of blood progenitor cells. These cells develop primarily in the bone marrow (BM), from which they can circulate to peripheral hematopoietic tissues. Leukemia causes certain systemic symptoms such as anemia and bleeding, and it increases the risk of infections that threaten human life [16]. The search for new therapeutic solutions encourages the use of tools

associated with nanotechnology. Metal nanoparticles possess excellent physical and chemical properties, which have been successfully utilized in several technological and biomedical fields [17]. For a long time, they have been applied in studies on various types of cancer, including liver [18], gastric [19], lung cancer [20] or glioma [21]. In the literature, there are information about the anticancer activity of metal nanoparticles such as: CuO [22, 23], ZnO [23] or Au [21, 24, 25]. In this work, anticancer effect of trimetallic Au/CuO/ZnO nanoparticles obtained from biological synthesis was studied. The cytotoxicity of Au/CuO/ZnO nanoparticles was measured by MTT assay. The impact of those nanoparticles on the viability of the Jurkat cell line was determined using ten model concentrations: 0.1; 0.5; 1; 2; 4; 6; 8; and

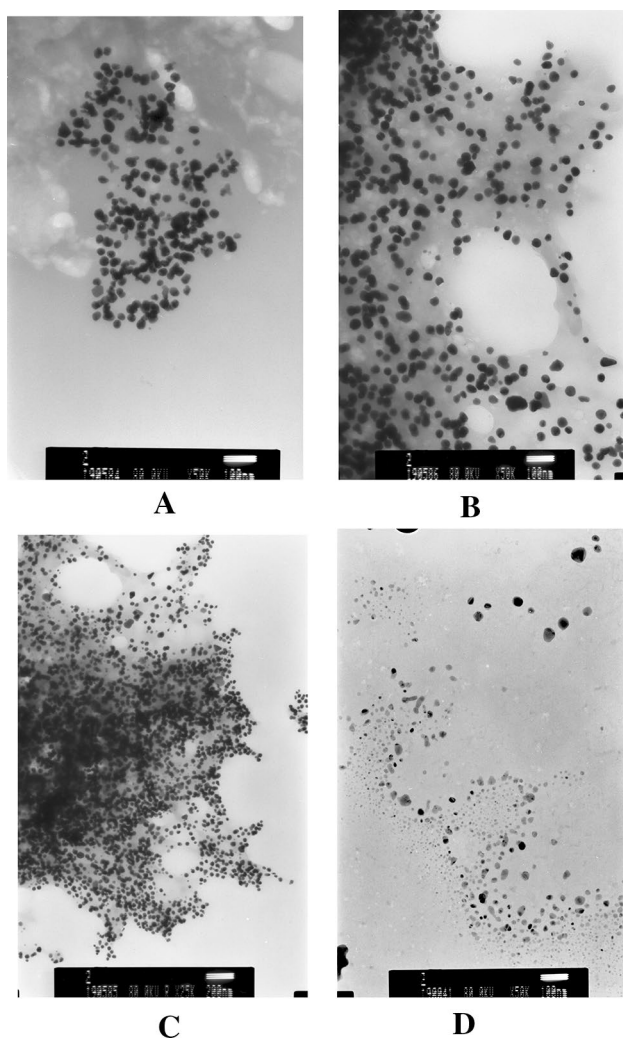


Fig. 6 TEM image of the Au/CuO/ZnO nanoparticles synthesized using *V. officinalis* extract

10 μmol . The cultures were carried out for 24, 48 and 72 h. Control cells were cultured without the addition of nanoparticles. The analysis showed that cell viability rapidly reduced in the range of 80–20% at the concentration of 0.1–4 μmol . At the concentration of 6 μmol , the relative cell viability value was close to zero. IC_{50} was 1.08 μmol for the culture carried out for 24 h; 0.64 μmol for 48 h; and 0.39 μmol for 72 h (Fig. 7).

3.5.2 Annexin V Binding Test

The Annexin V binding test was carried out to determine the mechanism of Jurkat cell elimination by the Au/CuO/ZnO nanoparticle complex. This test uses the ability of Annexin V to bind to phosphatidylserine residues externalized to cell membrane surface by apoptotic cells, and the penetration of cells by propidium iodide (PI) through the discontinuous cell membranes of necrotic cells. The simultaneous use of fluorochrome-labeled Annexin V and propidium iodide makes it possible to differentiate between viable and dead cells as well as identify cells in the state of early apoptosis, late apoptosis and necrosis (Fig. 8).

In order to perform the test, Jurkat cells were exposed to the complex of Au/CuO/ZnO nanoparticles at the concentration of 1, 10 and 100 μmol and assessed at 24 and 48 h of culture. The Annexin V binding test confirmed the previous results of the MTT assay, which indicate that the studied complex of Au/CuO/ZnO nanoparticles has a strong cytotoxic effect on the Jurkat cell line. The type of death and the effectiveness of cell elimination depended both on the concentration of the complex and the duration of culture. Within the first 24 h, over 80% of cells cultured in the presence of Au/CuO/ZnO nanoparticles at the concentration of 10 μmol exhibited the signs of late apoptosis, and about 60% of cells cultured in the presence of Au/CuO/ZnO nanoparticles at the concentration of 100

Au/CuO/ZnO - NP	
	IC_{50} (μM) \pm SD*
24h	1.07764 \pm 1.4740
48h	0.64518 \pm 1.1772
72h	0.39085 \pm 1.2995

* values are means \pm SD (n=3)

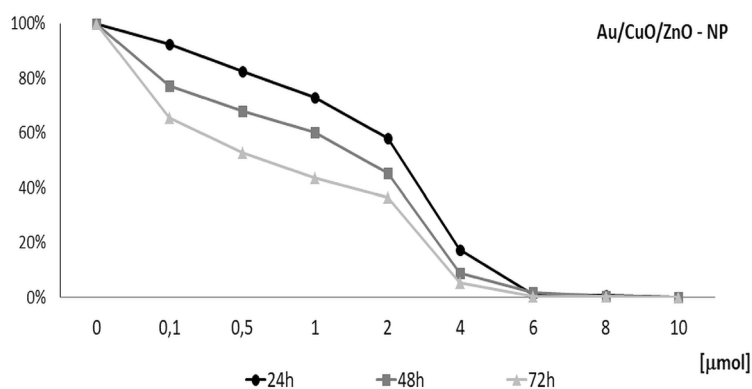
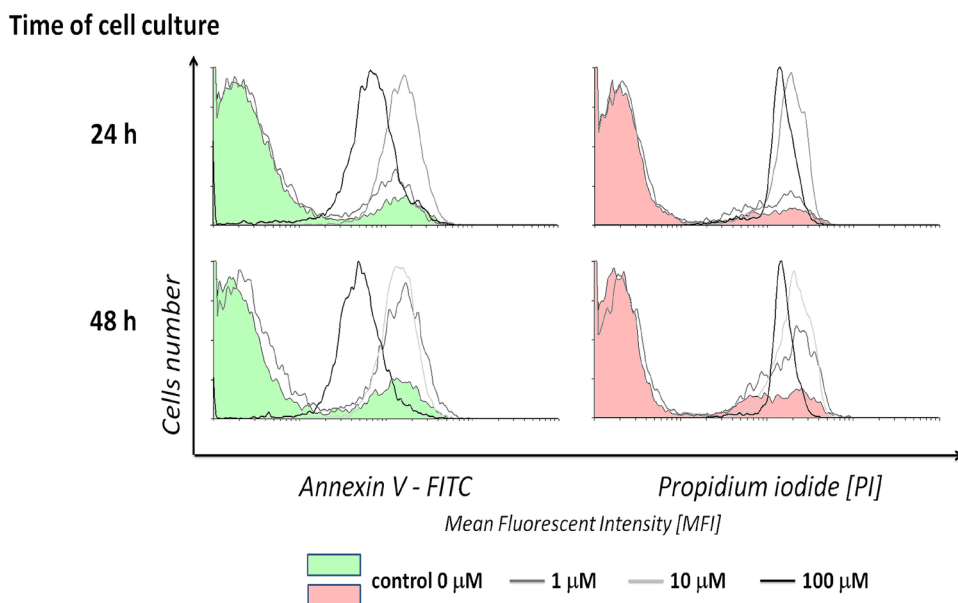


Fig. 7 Relative viability of the Jurkat cell line cultured for 24, 48 and 72 h in the presence of Au/CuO/ZnO nanoparticles at different concentrations. The table presents the IC_{50} values for different culture times

Fig. 8 Histograms of the Mean Fluorescence Intensity of FITC-conjugated Annexin V and propidium iodide (PI) during the Annexin V binding test carried out for Jurkat cells cultured in the presence of Au/CuO/ZnO nanoparticles at 24 and 48 h of culture



μmol underwent necrosis. At 48 h of culture, over 80% of cells cultured in the presence of the tested substance at the concentration of 100 μmol showed the signs of necrosis. When the culture time was extended to 48 h, even at the concentration of 1 μmol the amount of cells in the state of late apoptosis was about 50%. The mortality level of control cells during the test did not exceed 20% (Fig. 9).

3.5.3 Assessment of Cell Cycle

The cell cycle of the Jurkat cell line stimulated by Au/CuO/ZnO nanoparticles was assessed using the fluorescent properties of propidium iodide. For this purpose, cells were cultured for 24, 48 and 72 h with the addition of Au/CuO/ZnO nanoparticle complexes at the concentration of 1, 10 and 100 μmol (Fig. 10).

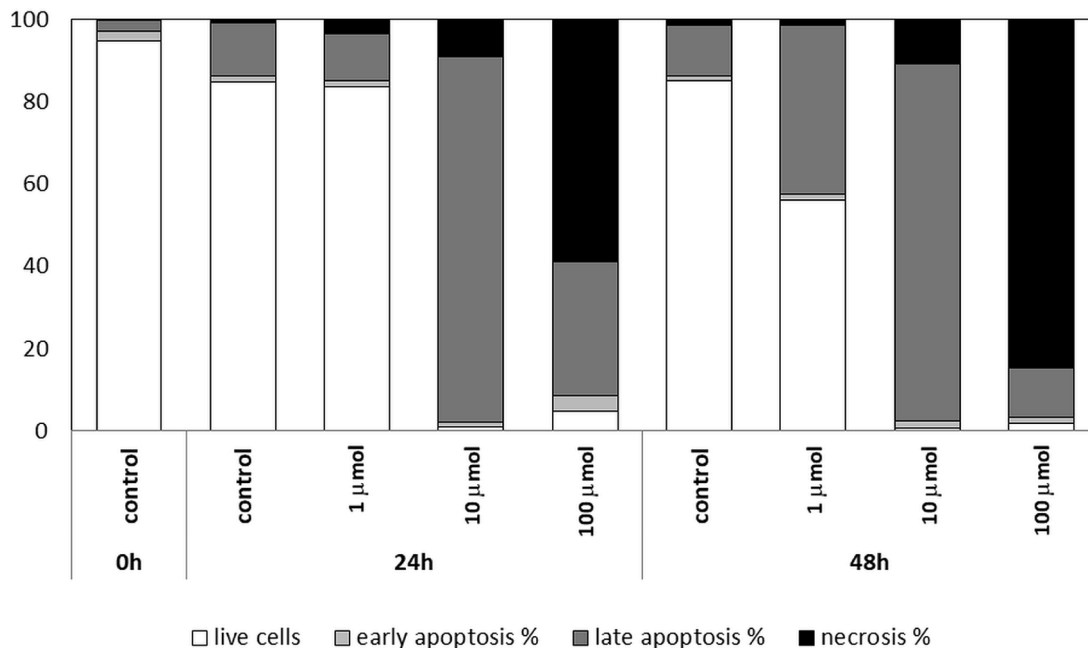


Fig. 9 Percentages of Jurkat cells cultured for 24 and 48 h in the presence of different concentrations of Au/CuO/ZnO nanoparticles synthesized using Verbena officinalis extract, showing a state of early apoptosis, late apoptosis and necrosis

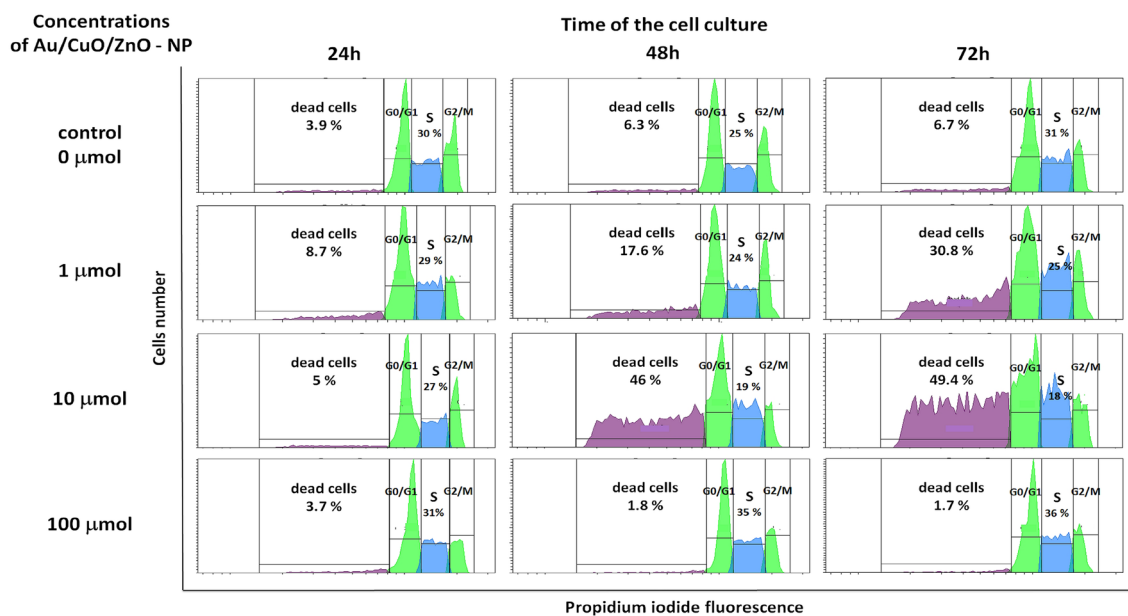


Fig. 10 Histograms of the respective cell cycle phases of the Jurkat cell line cultured for 24, 48 and 72 h in the presence of 1, 10 and 100 μmol of Au/CuO/ZnO nanoparticle complex

During the analysis, it was found that, similarly as in the case of the MTT assay and the Annexin V binding test, the percentage of dead cells increased with the concentration of Au/CuO/ZnO nanoparticles and the duration of culture. After 24 h, the amount of viable cells cultured in the presence of 100 μmol of the tested nanoparticles was so low that further analysis was considered unreliable (less than 2% viable cells). In the remaining samples, the highest percentage of dead cells was observed after applying the tested nanoparticles at the concentration of 10 μmol . At the 48th hour of culture, cell mortality caused by the tested complexes at the concentration of 10 μmol was 46%, and at the 72nd hour it reached 49%. As regards cells cultured in the presence of 1 μmol of the tested nanoparticles, their mortality considerably increased at 72 h of culture and reached about 31%. For cells treated with nanoparticles, the percentages of cells in the S phase and the G2/M phase were markedly lower than in the case of control cells. Such a rapid change in proportions between the S and G2/M phases and the percentage of dead cells is connected with the observation from the MTT test, which confirmed the high cytotoxicity of the tested nanoparticles, as well as the observation from the Annexin V binding test, which indicated a sudden death of Jurkat cells. (Fig. 11).

In addition, the study assessed the cell cycle of lymphocytes in PBMC separated from peripheral blood. In the course of the analysis, similarly as in the case of Jurkat cells, the percentage of dead cells among lymphocytes cultured in the presence of the tested nanoparticles was much higher than in the case of cells cultured without the addition of Au/

CuO/ZnO nanoparticles. The amount of viable cells cultured at the concentration of 100 μmol was very limited, which prevented a credible analysis. The concentration of 10 μmol caused a considerable increase in the percentage of dead lymphocytes, i.e. about 20% after 24 h, and over 30% after 48 h of culture (Fig. 12).

4 Conclusions

Green chemistry is emerging as a method for synthesizing various chemical products, including nanoparticles. This approach considerably mitigates the threat to the environment as it eliminates hazardous materials from preparation methods that are toxic to humans. Therefore, green synthesis of metal nanoparticles creates a better platform for synthesizing various nanomaterials, including trimetallic nanoparticles. In this work, Au/CuO/ZnO nanoparticles were synthesized using *V. officinalis* extract. The research methods applied in the study, including UV-VIS, FT-IR, TEM and AFM methods, confirmed the presence of spherical trimetallic nanoparticles of about 35 nm. The type of death and the effectiveness of cell elimination depended both on the concentration of the complex and the duration of culture. Within the first 24 h, over 80% of cells cultured in the presence of Au/CuO/ZnO nanoparticles at the concentration of 10 μmol exhibited the signs of late apoptosis, and about 60% of cells cultured in the presence of Au/CuO/ZnO nanoparticles at the concentration of 100 μmol underwent necrosis. The present intense studies that employ metal nanoparticles may lead to the emergence of new medical

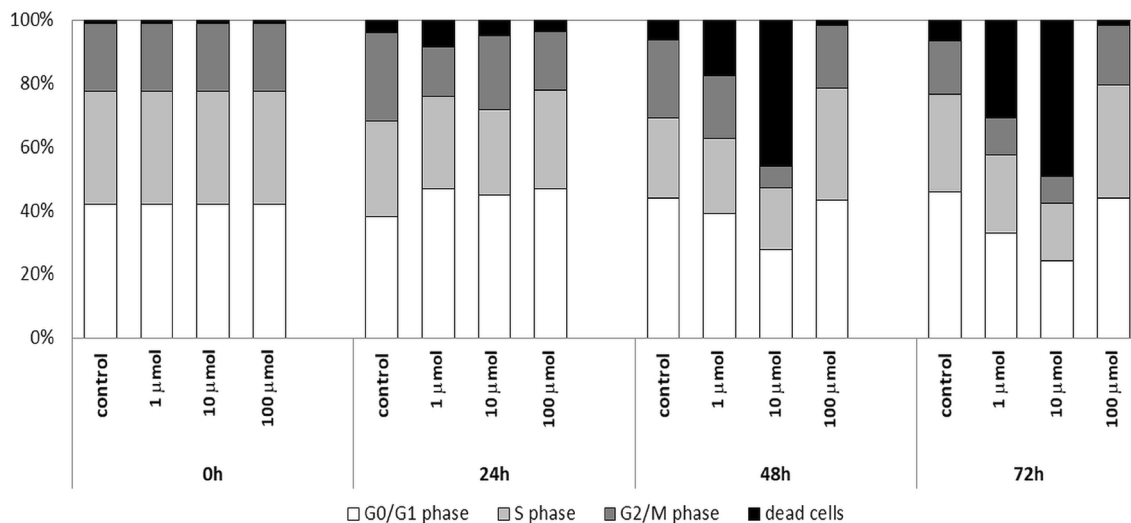
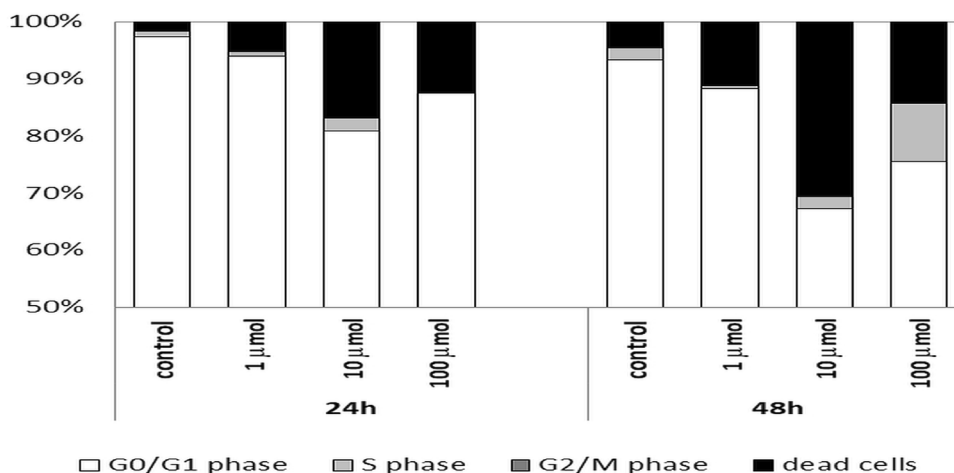


Fig. 11 Percentage of the respective cell cycle phases of Jurkat cells cultured in the presence of 1, 10 and 100 μmol of Au/CuO/ZnO nanoparticle complex for 24, 48 and 72 h

Fig. 12 Percentage of the respective cell cycle phases of lymphocytes in the tested PBMC samples cultured in the presence of 1, 10 and 100 μmol of Au/CuO/ZnO nanoparticle complex for 24 and 48 h



solutions to various kinds of cancer. The conducted studies proved that trimetallic Au/CuO/ZnO nanoparticles obtained from biological synthesis can help to develop effective solutions to combat leukemia.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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