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Bio-mediated synthesis, characterization and cytotoxicity of gold nanoparticles.

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We report here a "green" approach for the synthesis of gold nanoparticles (GNPs) in which the *Mentha piperita* extract was applied for bioreduction of chloroauric acid and stabilization of the formed nanostructures. The obtained GNPs were characterized by UV-Vis absorption spectroscopy and transmission electron microscopy (TEM). Reduction of gold ions with the plant extract leads to the production of nanoparticles with various shapes (spherical, triangular and hexagonal) and sizes (from 10 to 300 nm). The kinetics of the reaction was monitored and various conditions of the synthesis were investigated. As the result, we established protocols optimized towards the synthesis of nanospheres and nanoprisms of gold. The cytotoxic effect of the obtained gold nanoparticles was studied by performing MTT assay, which showed lower cytotoxicity of the biosynthesized GNPs compared to gold nanorods synthesized using the usual seed-mediated growth. The results suggest that the synthesis using plant extracts may be a useful way to produce gold nanostructures for various biological and medical applications.

Introduction

Noble metal nanoparticles have attracted much attention due to their unique size and shape dependent physical and chemical properties[1]. These features make them interesting for use in many fields of science and technology (e.g., electronics[2], optics[3] and medicine[4]). Various nanoparticles with well-defined chemical composition, size, and morphology can be synthesized using chemical[5] and physical[6] methods, however, many of those techniques are quite expensive and/or involve the use of hazardous chemicals. Biological synthesis employing microorganisms[7], fungi[8] or plants[9] is an alternative method to produce nanoparticles in a low-cost, simple and eco-friendly way. The potential utility of the nanoparticles in medical and biological applications prompted intensification of the studies on the toxicity of these structures. Various features of the GNPs were taken into account in toxicological tests, such as size, shape[10] and stabilizing agents[11]. Unfortunately, it is difficult to draw general conclusions, because of the variability of the applied test conditions (including cell type, culturing conditions and dosing)[12]. Despite these limitations it has become evident that the coating of the gold nanoparticles plays an important role in their cytotoxicity[13].

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We report here the biosynthesis of gold nanoparticles (GNPs) using mint (*Mentha piperita*) extract and their characterization. *Mentha piperita* (*M. piperita*) is a popular herb belonging to the family Lamiaceae and commonly known as peppermint[14]. Various forms of peppermint (including oil, spirit and aqueous extracts[15]) are widely used as an ingredients in pharmaceutical products[16], cosmetics and foods[17]. In our work the GNPs were obtained using the aqueous extract of *M. piperita*. It contains both reducing and stabilizing agents involved in the process of the formation of gold nanoparticles from tetrachloroauric acid solution and there is no need to add any other components to the reaction mixture. The studies of cytotoxicity of the obtained nanoparticles proved that these structures were coated with non-toxic agents and thus might be potentially useful in biomedical applications.

Materials and methods

Reagents

Tetrachloroauric(III) acid ($HAuCl_4 \cdot 3H_2O$) was obtained from Sigma-Aldrich and used as received. Dried, powdered leaves of *Mentha piperita* were purchased in a local market.

Preparation of mint extract

The extract was prepared by taking 5 g of dried, powdered leaves of *Mentha piperita* along with 50 ml of distilled, deionized water. The mixture was kept in water bath at 100°C for 30 min and then centrifuged at 5000 rpm (MPW-380 Centrifuge) for 15 min. The supernatant was filtered through

filter paper. The obtained solution was collected and stored at 4°C for further experiments.

Synthesis of gold nanoparticles using mint extract

For the synthesis of gold nanoparticles the protocol of MubarakAli et al.[18] was applied. Briefly, 250 µl of mint extract was added to 5 ml of 1 mM chloroauric acid aqueous solution. The reaction was carried out in dark, at 28°C for 24 h. Change of the color from yellow to ruby red indicated the presence of gold nanoparticles in the solution. The mixture was centrifuged at 5000 rpm (MPW-380 Centrifuge) for 10 min and the pellet was resuspended in distilled, deionized water. The solution of GNPs was stored at 4°C.

Characterization of gold nanoparticles

The obtained gold nanoparticles were characterized by UV-Vis absorption spectroscopy (JASCO V-670 Spectrophotometer) and transmission electron microscopy (TEM) (FEI Tecnai G2 20 X-TWIN). UV-Vis spectra were measured in 10 mm quartz cuvettes at room temperature in 400-1300 nm range. Samples for high-resolution transmission electron microscopic analysis were prepared by dropping of the solution of GNPs onto carbon coated copper grids. The excess of the solvent was removed by evaporation.

Fractionation of mint extract

To identify the molecules responsible for the reduction and the stabilization of the formed gold nanoparticles, different fractions of the extract were separated according to the molecular weight of its components. The first fraction was obtained by centrifugation of the extract at 6000 rpm (MPW-380 Centrifuge) for 10 min and resuspension of the pellet in distilled, deionized water. The second and the third fractions were prepared using the supernatant from the previous step. Filter units (Amicon Ultra-4 3K) were applied for the further separation. After 30 min of centrifugation at 7000 rpm (MPW-380 Centrifuge) two fractions (components with molecular weight lower than 3 kDa and components with molecular weight higher than 3 kDa) were collected. Finally, the biosynthesis of gold nanoparticles using each fraction was examined.

Separation of GNPs using sucrose density gradient

Separation of the GNPs was carried out in sucrose density gradient, prepared by layering aqueous sucrose solutions with decreasing sucrose concentration[19]. 2 ml of each of sucrose solutions were applied successively upon one another: 70%, 60%, 50%, 40% and 30% w/v sucrose solution. At the top of the gradient 2 ml of GNPs mixture was loaded (Fig. 1).

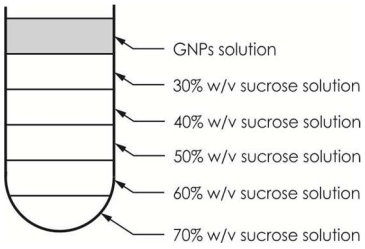

Fig. 1. Scheme of a sample prepared for centrifugation.

After 40 min of centrifugation at 4700 rpm (Thermo Heraeus Multifuge X3R Centrifuge), the fractions of the gradient were collected (2 ml each). In the next step gold nanoparticles from individual fractions were purified by centrifuging and resuspending in distilled, deionized water and characterized by UV-Vis spectroscopy and TEM.

Cytotoxicity of gold nanoparticles

To study the cytotoxic effect of gold nanoparticles synthesized using mint extract the MTT assay was performed. It is a colorimetric, enzyme-based assay, widely used for assessing cell viability in chemosensitivity testing[20]. MTT is a tetrazolium salt, which is converted into purple colored formazan product with an absorbance maximum near 570 nm[21]. The principle of the MTT assay utilizes the reduction of MTT by succinate dehydrogenase (a mitochondrial enzyme), which is active only in metabolically viable cells. For most living cells the mitochondrial activity is constant and the number of viable cells is directly related to the formazan production. Consequently, the cell viability can be monitored by detection of the changes of formazan concentration[22].
In our research the following parameters of the assay were applied. MTT (Sigma M5655) was dissolved in medium (Opti-MEM, Life Technologies) to the concentration of 5 mg/ml, filtered through a 0.2 µm filter and stored at 2-8°C. Hek293 (ATCC) cells were seeded (2.25 x 10^6 cells/plate) in 96-well plates and grown for 24 h. The cells were then treated with nanoparticles for 24 h. Next the MTT stock solution was added to each well in volume equal to one-tenth of the original culture volume and incubated for 4 h. Finally, the medium was removed and cells were lysed in 0.1 M HCl solution (in isopropanol). Absorbance of converted dye was measured at 570 nm with background subtraction at 630 nm.

Results and discussion

Biosynthesis of gold nanoparticles

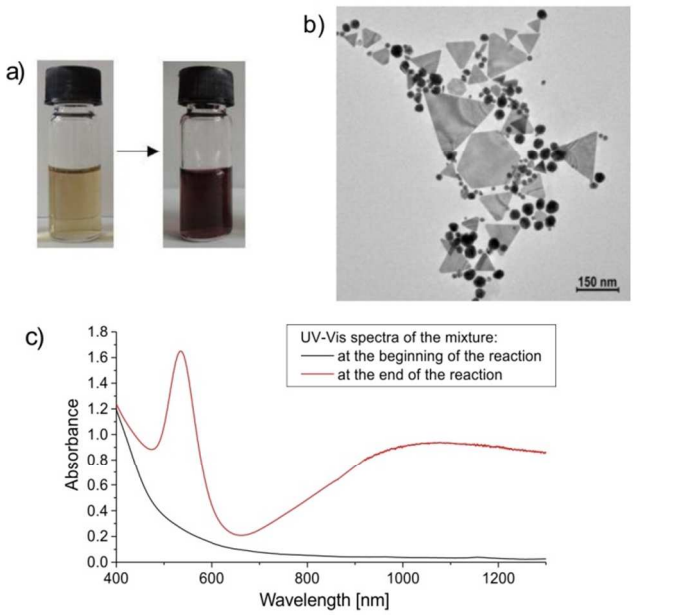

Fig. 2. a) Colors of the mixture at the beginning (left) and at the end (right) of the reaction; b) TEM image of the synthesized GNPs; c) UV-Vis spectrum of the obtained nanoparticles.

The addition of mint extract to $HAuCl_4$ aqueous solution results in the synthesis of gold nanoparticles. This reaction can be easily followed by visible change of color (Fig. 2a) and UV-Vis spectroscopy due to the emergence of characteristic surface plasmon resonance (SPR) bands of gold nanoparticles[23]. The UV-Vis spectrum of the obtained GNPs (Fig. 2c) reveals two SPR bands: the narrow one at 540 nm and the broad one at 900-1000 nm indicating the presence of anisotropic shapes (triangular and hexagonal) of nanoparticles[24]. TEM image shows the morphology of the formed nanoparticles (Fig. 2b). The biosynthesis leads to the production of highly polydisperse nanoparticles with various shapes and sizes (from 10 to 300 nm). The obtained nanoparticles mixture contained 90% of nanospheres, 8% of nanoprisms and 2% of hexagonal nanoplates. Consequently, we can produce gold nanoparticles applying green chemistry approach, where different components of the mint extract may cause the formation of nanoparticles with different morphologies.

Kinetics of the reaction

The kinetics of the reaction of synthesis of gold nanoparticles was examined by recording the changes of the UV-Vis spectra in time (Fig. 3a). Two wavelengths, corresponding to the maxima of the SPR bands, were chosen for further analysis: 540 nm and 900 nm. With the progress of the reaction the first peak increases continuously reaching its maximum after 2 h (Fig. 3b). The second peak becomes visible after 10 min and increases with time, indicating appearance of anisotropic nanostructures (Fig. 3c).

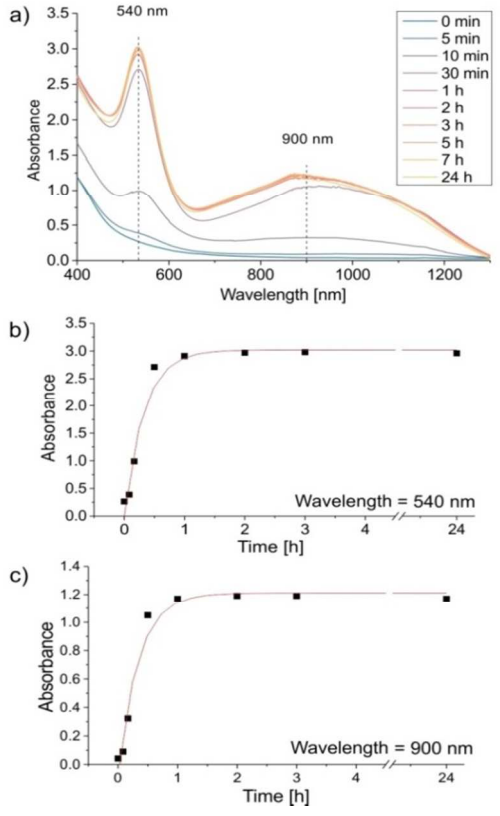

Fig. 3. a) UV-Vis spectra of GNPs recorded in time; Absorption at b) 540 nm and c) 900 nm as function of time with the fitting to the first-order rate law.

Additionally, formation of different shapes of GNPs in reaction mixture was monitored using TEM. Initially, only spherical nanoparticles were observed (Fig. 4a) but with the progress of the reaction flat, anisotropic nanoparticles appeared (Fig. 4b, 4c). The population of hexagonal and triangular GNPs increased with time until the reaction was terminated after 2 h.

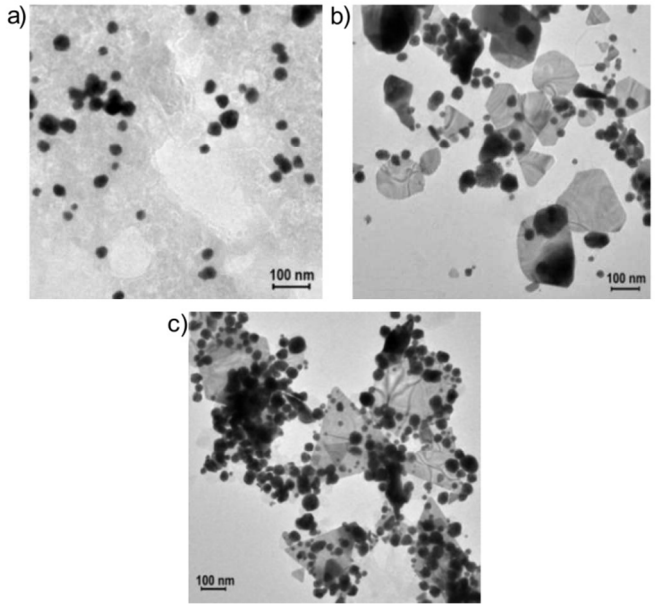

Fig. 4. Formation of GNPs after a) 10 min ; b) 30 min and c) 1 h.

We suppose that small spheres formed at the beginning of the reaction may play a role of seeds, which grow under different local conditions forming different shapes of the nanoparticles.

Synthesis of gold nanoparticles under different pH conditions

The influence of pH on the synthesis yield and the shape of GNPs was studied by carrying out the reaction under different pH conditions. The pH was adjusted with 0.1 M NaOH or 0.1 M H_2SO_4. Seven reaction mixtures were prepared: at pH equal to 2, 4, 6, 8, 10, 12 and the control without changing the pH (initial pH of 5.5). Fig. 5 presents the UV-Vis spectra of the obtained nanoparticles, measured after centrifugation and resuspension in distilled, deionized water.

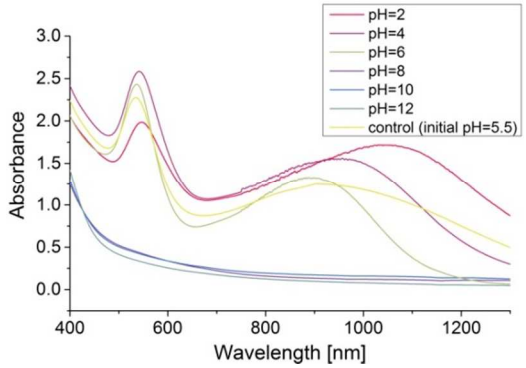

Fig. 5. UV-Vis spectra of gold nanoparticles synthesized under different pH conditions.

The decrease of the pH promotes the formation of gold nanoparticles, especially with anisotropic shape. Alkaline conditions (pH higher than 8) inhibit the synthesis. It is likely that at the high pH some crucial components of the extract are damaged or some necessary interactions are prevented, causing the inhibition of the production of the GNPs.

Biosynthesis using different fractions of the mint extract

The synthesis of gold nanoparticles using different fractions of the extract was carried out following the protocol described in "Materials and methods" section, replacing the whole extract with one of the fractions. The reaction using the whole extract was performed as the control. Fig. 6 shows the UV-Vis spectra of the solutions after 24 h of the reaction.

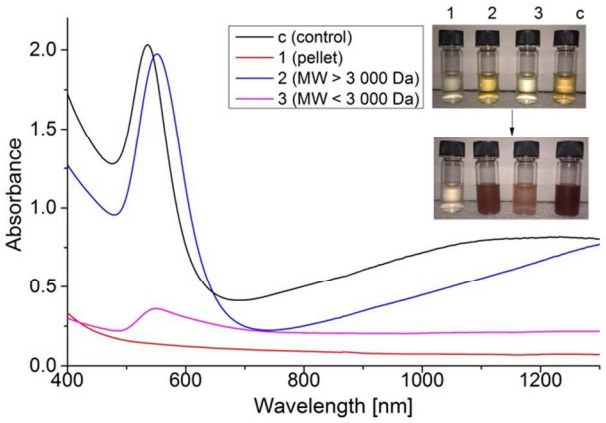

Fig. 6. UV-Vis spectra and visible change of color of the solutions during synthesis of GNPs using different fractions of the extract.

For the first reaction mixture, containing the fraction obtained after centrifuging the extract and resuspending the pellet, no SPR band is observed, indicating that the components of this fraction are not involved in the synthesis of gold nanoparticles. Use of the second fraction (components with molecular weight higher than 3 kDa) for the synthesis leads to the production of GNPs, similarly to the whole extract, while the synthesis using the third fraction (components with molecular weight lower than 3 kDa) proceeds with significantly lower yield. These results indicate that the most important reducing and stabilizing agents are included in the second fraction. It is consistent with the assumption that some proteins may be involved in the synthesis, because most of these macromolecules have molecular weight higher than 3 kDa.

Further analysis of the molecules involved in the synthesis

We have performed additional experiments (phosphorous and proton NMR measurements and electrophoresis of the extract and of the obtained nanoparticles) to identify the molecules involved in the synthesis of gold nanoparticles, but we could not find the exact components responsible for this process. There were no signals on the phosphorous NMR and the signals on the proton NMR were overlapped (Supplementary Information, Fig. 1). We obtained similar results after the electrophoresis, which revealed a lot of proteins present at the surface of the obtained GNP (Supplementary Information, Fig. 2).

We suppose that some proteins may stabilize the surface of formed nanoparticles, but not necessarily definite ones or in their native form (boiling of the extract or degradation of the proteins into smaller molecules using proteases does not disturb the synthesis). It seem that only some specific parts or functional groups are needed but not the proteins as the complete molecules.

Separation of GNPs using sucrose density gradient

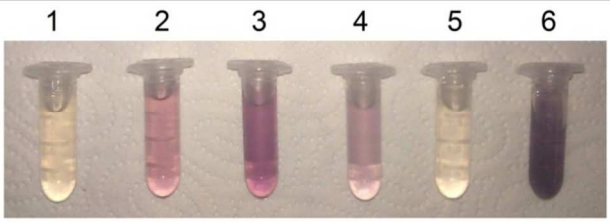

Fig. 7. Picture of fractions collected from the lowest to the highest sucrose concentration.

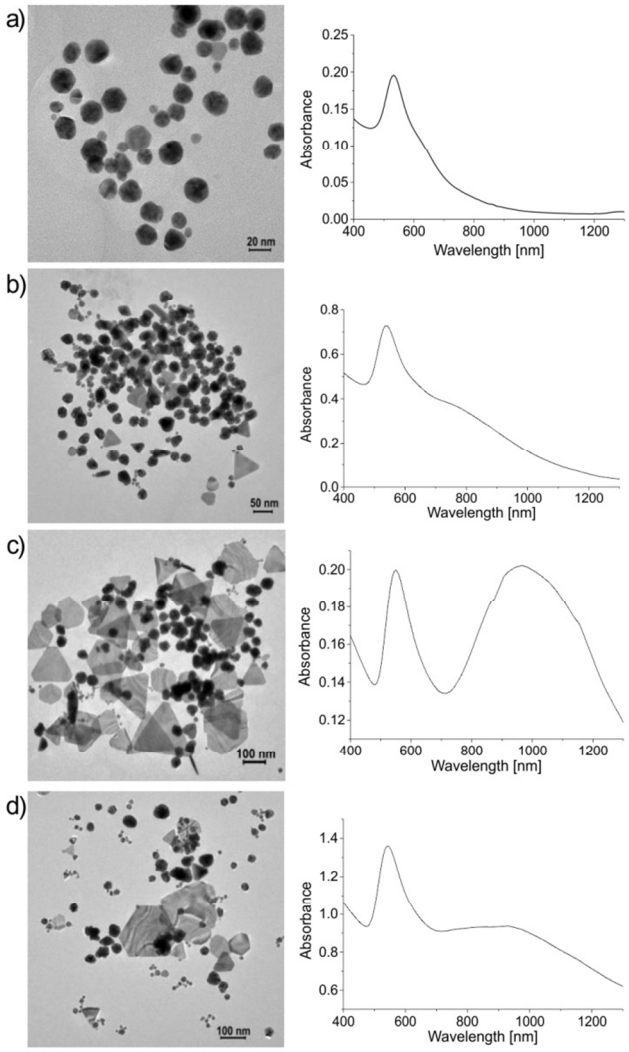

Fig. 8. UV-Vis spectra and TEM images of gold nanoparticles contained in: a) 2nd fraction; b) 3rd fraction; c) 4th fraction and d) 6th fraction.

Centrifugation in sucrose density gradient was applied for separation of different sizes and shapes of gold nanoparticles from the mixture obtained by the synthesis using mint extract carried out at the pH of 2. After centrifugation six fractions were collected (Fig. 7) and four of them (2, 3, 4 and 6) were taken for the further analysis. Fractions 1 and 5 contained insignificant concentrations of the GNPs.
The second fraction was mainly comprised (98%) of the smallest spherical nanoparticles (up to 30 nm) (Fig. 8a). The third fraction consisted of 94% of larger spheres (up to 50 nm) and a few (5%) small nanoprisms (Fig. 8b). The fourth fraction contained numerous flat, anisotropic structures (30%) along with the largest spherical nanoparticles (Fig. 8c). In the last fraction numerous aggregated nanoparticles with various sizes and shapes were observed (Fig. 8d).
Consequently, we increased the amount of triangular and hexagonal nanoparticles in the mixture from 10% to 30%. These structures are especially interesting for us mostly because of their optical properties.

Cytotoxicity of gold nanoparticles

The cytotoxic effect of the gold nanoparticles was examined by performing MTT assay. Five samples of GNPs were tested: the mixture obtained after the synthesis using mint extract carried out at the pH of 2 (crude product and additionally purified by centrifugation), the 2nd and the 4th fraction obtained from the separation in sucrose density gradient and gold nanorods synthesized by seed-mediated growth[25]. At first the toxicity of gold nanoparticles synthesized using mint extract was compared with the toxicity of gold nanorods. Next the cytotoxic effect of the GNPs from the 2nd and the 4th fraction was examined. The cells were incubated with the nanoparticles at the final concentration of 100 µg/ml. It can be noticed that the biologically synthesized nanoparticles are less toxic than those synthesized using the chemical method (Fig. 9). Additionally, the purified nanoparticles synthesized using mint extract (five times centrifuged and resuspended in distilled, deionized water) are almost nontoxic. High cytotoxicity of gold nanorods results mostly from the capping agent, CTAB[11], commonly used for the chemical synthesis of gold nanoparticles. The presence of the free surfactant in the solution is needed to provide the stability of the gold nanorods. Consequently, the cytotoxic effect cannot be decreased by centrifugation and resuspension of the nanorods. By contrast, the GNPs synthesized using mint extract are coated with biological agents, occurring in the plant, ensuring low cytotoxicity.

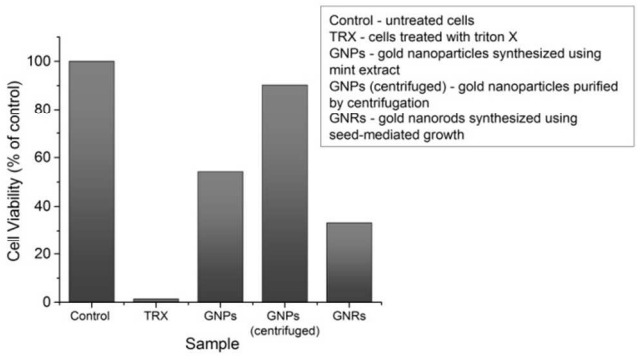

Fig. 9. Cytotoxic effect of GNPs measured by MTT assay.

Fig. 10 presents the influence of the morphology of gold nanoparticles on the cytotoxicity. The results show that the mixture containing increased amount of flat, anisotropic structures (4th fraction) is slightly more toxic comparing with the second fraction consisting mainly of spherical nanoparticles. However, observed difference in the cytotoxicity of the GNPs from examined fractions is not as significant as in the case of the biologically and chemically synthesized nanoparticles. Consequently, it can be assumed that the size and the shape of the gold nanoparticles does not affect the cytotoxicity as much as the capping agent.

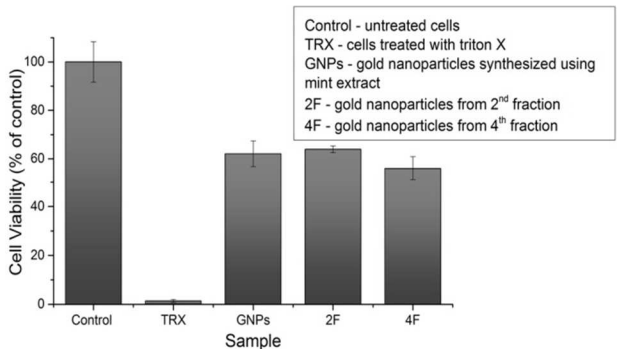

Fig. 10. Cytotoxic effect of gold nanoparticles separated in sucrose density gradient.

To conclude, we synthesized gold nanoparticles using plant extract as a source of reducing and stabilizing agents, which provides formation of the GNPs coated with nontoxic, biological molecules.

Conclusions

The reported procedure is a simple, economical and environmentally friendly method to synthesize gold nanoparticles under mild conditions without using any harmful agents. The biological synthesis of GNPs using extract of *M. piperita* leaves results in the formation of nanostructures with various shapes (mainly spherical, triangular and hexagonal) ranging from 10 to 300 nm in size. The morphology and size of nanoparticles can be controlled to some extent by changing pH or time of the reaction. Additionally, some nanostructures can be separated from the mixture obtained after the synthesis by the centrifugation in sucrose density gradient. The MTT assay revealed promising results of low cytotoxic effect of GNPs synthesized using mint extract compared to chemically synthesized gold nanorods. Consequently, we conclude that biosynthesis may be a useful method to produce nanostructures potentially useful in biomedical, diagnostic and pharmaceutical applications.

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