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FACILE, LOW-COST AND RAPID PHYTOSYNTHESIS OF STABLE AND ECO-FRIENDLY GOLD NANOPARTICLES USING GREEN WALNUT SHELL AND STUDY OF THEIR ANTICANCER POTENTIAL

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Abstract – *Objective:* *Nowadays, gold nanoparticles (GNPs) are used in targeted nano photothermal cancer therapy. Considerable interest has been dedicated to gold nanoparticles because of their characteristics which are controllable and unique. Different synthesis methods have been proposed to produce these nanoparticles, which often require elevated temperatures/pressures or toxic solvents. Therefore, green synthesis would be a substitution selection as an environmentally friendly, economically viable and simple alternative method for the gold nanoparticles synthesis.*

Materials and Methods: *In this study, using walnut green external shell, GNPs have been synthesized by green chemistry method. In this reaction, walnut green shell is a reducer and stabilizing factor for preparing GNPs. In this work, after extracting walnut, 2 ml of extract was added to 4 ml of Au+3 solution ( ), purple color indicates synthesis of GNPs. For NPs synthesis with an appropriate size, some factors like pH, extract volume, gold salt concentration and reaction temperature were surveyed and by using UV-Visible (UV-Vis) absorption spectroscopy, optimum conditions were selected for preparing NPs. In addition, prepared NPs have been tested by x-ray diffraction (XRD) and transmission electron microscopy (TEM) to determine structure, size and their shape. Afterward, the synthesized GNPs were determined by 3- (4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) to assay their antitumor, anti-oxidant properties.*

Results: *The results of these measurements show the spherical and triangular GNPs with different sizes between 10-50 nm were produced. There is an important development in the antioxidant and cytotoxicity characteristics of GNPs which are green synthesized ones. Statistical analysis also found a significant difference among various GNPs concentrations on declined cell viability of Michigan Cancer Foundation-7 (MCF7) cells in 24 h in concentration-dependent fashion. The IC50 value evaluated after 24 h of GNPs against MCF7 cells caused 52% cell death at the concentration of 3 mM.*

Conclusions: *The above-mentioned results suggest that synthesized nanoparticles employing green nanotechnology is a suitable technique to fight against infectious diseases and cancer.*

KEYWORDS: *Anticancer potential, Green synthesis, Gold nanoparticles, Phytosynthesis, Selective cytotoxicity.*

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INTRODUCTION

Nanotechnology, in developing countries, is being applied to assist disease treatment and is a kind of prevention for health concerns. For that sort of nanotechnology, nanomedicine is the umbrella term. Additionally, nanotechnology is being used or improved toward industrial diversity, and purified NP is applied in the various manufacturing products and processes[1,2], and healthcare involving lubricant additives, filters, paints, and insulation. The novel drugs for age are the NPs of ceramics, metals, or polymers that can fight conditions such as cancer[3] and combat human pathogens such as bacteria[4]. Nanotechnology usages in the control, diagnosis, treatment, and monitoring of diseases have been attributed to nanomedicine. NPs are positive and helpful agents in cancer therapy and are analyzed as radio-sensitizers, drug carriers, contrast agents, and photo-thermal agents[4]. Also, as regards the high activity of NPs and their tendency to connect with biomolecules and macromolecules, the NPs linked to peptides can be used as detectors inside molecules, which is a good and important method in cellular pictures, drug delivery and biomolecules recognition[5]. Gold NPs are functionalized by different biological factors and are recognized and detected after interring in the body and transferring to the target section. Toward the areas of special disease, GNPs external function for biomedical application is essential for targeting them and allows interacting selectively with biomolecules or cells. In cancer therapy, one of the main and significant uses is the gold NPs. Studies indicate solid gold nanospheres selectively kill cancer cells and have no adverse effects on the healthy cells[6,7]. NPs are synthesized by two methods: 1) Chemical and physical methods that have more yield, produced NPs in this method are purer, but chemical materials in synthetic NPs are toxic and pollute the environment. 2) Green method that uses biological factors to synthesize NPs. This method uses plants to produce NPs and it is a safe and suitable method. Cells are randomly divided by cancer. This damages the immune system, further impairment, and tumors which are fatal. In many developed countries among females[8], breast cancer has been recognized as the conducting cause for cancer death. Also, in less developed countries, among females, it remains the leading cause of cancer death. Every year 1.7 million people are afflicted with breast cancer[9]. The most common cause of death is a metastatic disease to various organs[10]. In general, most of the chemical drugs that are used in cancer therapy change the meiosis process and stop cancer cell diagnosis. This happens in different ways such as apoptosis induction, DNA structure alteration, tyrosine kinase, and so on[11]. Regarding the fact that chemical drugs have high toxicity and destroy safe cells, many efforts have been made to use drugs that have the most effective and the lowest-side effects. Even though in the diagnosis and treatment of cancer, considerable progress has been made, but the eradication of it is limited. As a result, finding novel treatment and diagnosis approaches is crucial[12,13]. Medically, nanotechnology for cancer is an emerging field that aims to develop advancements toward both the treatment and diagnosis of cancer. Much attention has been paid to gold nanoparticles, because of the biocompatibility of them, their better optical properties, and they have capability to chemically modify the surface of them via the addition of numerous types of ligands[14]. Concerning the importance of gold NPs in cancer therapy, in this research, experimental studies were done to synthesize gold NPs of walnut green shells in order to cure breast cancer. Walnuts are a rich source of multiple nutrients and are cultivated in different areas of the world[15]. In this study, the walnut green shell was used to synthesize gold NPs and their anticancer and antioxidant properties were recognized by MTT assay and these NPs were applied in cancer treatment.

MATERIALS AND METHODS

Extraction

First, a walnut green shell was prepared which is a disposable part of the plant, the shells were washed and dried in a dark room. After this, they were ground and weighted to 1 gr of dried powder and dissolved in 100 mL deionized water. This solution was placed on the shaker at 50°C for 30 min. After cooling the solution, it was filtrated using Whatman filter paper No. 42. Finally, for deletion of suspended particle, the extract was put in centrifuge with 1000 revolutions per min for 30 min. The extract was prepared and kept at a temperature of 4°C[16].

Synthesis of gold NPs

For the synthesis of gold NPs, the first 1 mM concentration of $HAuCl_4.3H_2O$ was prepared and 4 ml of this solution was added to 2 ml walnut extract at room temperature. The measured pH was 5.4. Color solution change from green to purple showed synthesized gold NPs.

Optimum conditions for producing bigger gold NPs

In order to make gold NPs with high quality and bigger size, we changed some factors like pH, extract volume, gold solution concentration, and temperature.

Optimum pH

We prepared six solutions (2 ml walnut green shell extract + 4 ml gold solution 1 mM) with different pH between 3-8. pH setting was done by 0.1 M (Hydrogen chloride) HCl and 0.1 M (Sodium hydroxide) NaOH. By comparing the spectrum of these solutions, optimum pH was selected.

Optimization extracts volume

In the next step, to select the best walnut extract volume, 4 ml of gold solution was added to four tubes and 1-4 ml of extract was added to each tube, respectively. pH in each tube was optimum pH. Based on the absorption spectrum of different solutions, volume was selected.

Optimization of gold salt concentration

In this step, five different concentrations of gold solution (0.5, 1, 2, 3, 4 mM) were prepared and at optimum pH, optimum volume of extract was added to each tube. The maximum absorption shows the optimum concentration of the gold solution.

Optimum temperature

In the final step, four solutions with the mentioned optimum conditions were prepared and one of them was placed at room temperature (25°C), the other solutions were placed in a heat bath at different temperatures (35, 55, 75°C). Analyzing the solutions, the optimum temperature was determined.

Culture of MCF7 cells

A sample of MCF7 cells was purchased (from the National Cell Bank of Iran, Pasteur Institute). Cells were seeded into 90% RPMI-1640 which was inoculated with 10% heat-inactivated fetal bovine serum (Gibco Laboratories; Waltham, MA, USA) and contained Penicillin 100 IU/ml and streptomycin 100 µg/mL, with 5% CO_2 - 95% O2 in a 37ºC humidified incubator[17].

MTT assay

In medium culture, seeding density cells were plated on 96-well plates, and incubated at 37°C in humidified atmosphere including 5% carbon dioxide to allow cells to be added into the wells. After 24 h, cells were subjected to differing concentrations for the GNPs (0.5-5 mM) and were incubated at 37°C for 24 h. The cells with MTT were treated after exposure to the drug dissolved in PBS and incubated at 37°C for more than 4 h. Afterwards, with a syringe and needle, the whole liquid was extracted; and to each well, dimethyl sulfoxide was attached to dissolve the crystals of MTT-formazan. The plates were placed in a spectrophotometer which is a multi-well scanning. Then, every plate was quantified photo-metrically after stirring for the 30s, at 570 nm absorbance by ELISA reader (BioTek; Winoosky, VT, USA). All experiments were repeated 3 times. The purple formazan amount generated through treated cells was compared to that of cells that were untreated control, and in the treated cells, the absorbance was declared as a control percentage[17,18].

Instrumental analysis

Excel and SPSS software version 18 (SPSS Inc., Chicago, IL, USA) was used to analyze the data statistically. All experiments were executed in triplicate and all attained consequences, as the mean ± scanning electron microscope (SEM) (Germany). Also, data were investigated using one way ANOVA and ($p \leq 0.05$) was considered significant. To measure the size of synthesized NPs and study their shape, Transmission Electron Microscopy (TEM), (model Zeiss-EM10C-100 KV; Germany) was used. The results confirm that spherical and triangular gold NPs with the size between 10-50 nm have been synthesized. Furthermore, in order to identify the phase of crystalline NPs and for provision of information on unit cell dimensions, x-ray diffraction ((X-ray powder diffraction) XRD (Shimadzu; Vietnam Tube: Cu (Kα = 1.54 Å) X'Pert Pro MPD (PANalytical)) was applied.

RESULTS

Study of absorption spectroscopy of the Au NPs

Gold NPs were prepared by adding 4 ml of 1mM Au^{3+} solution to 2 ml walnut extract. After some minutes, the green-color solution changed to purple color that shows synthesized gold NPs, purple color is related to surface Plasmon resonance of gold NPs. Then, absorption spectrum of NPs and walnut extract were studied. Using visible-ultraviolet spectrophotometer in 200-800 nm gold NPs spectrum, maximum was determined 480 nm (Figure 1).

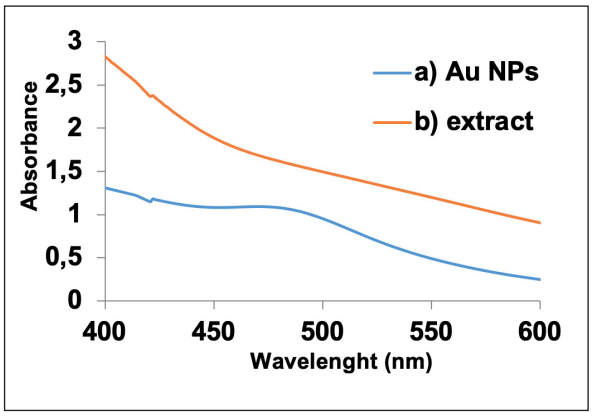

Fig. 1. UV- Vis absorption spectroscopy a) Au NPs and b) walnut green shell extract without optimum conditions.

Results of optimum pH

Six solutions with different pH between 3-8 were studied. By comparing the spectrum of these solutions, it was demonstrated that solution with pH=6 has maximum concentration. Thus, optimum pH is 6 (Figure 2).

Results of optimum extract volume

As mentioned before, to study optimum extract volume, 4 ml of gold solution was added to four tubes and extract volume was set between 1-4 ml for each tube, respectively. pH in each tube was 6 (optimum pH). Absorption spectrum of different solutions showed that more gold NPs were made in 2 ml extract volume. Thus, 2 ml was considered as optimum volume of extract (Figure 3).

Results of optimization of gold salt concentration

In this step, five different concentrations of gold solution (0.5, 1, 2, 3, 4 mM) were prepared with pH=6 and

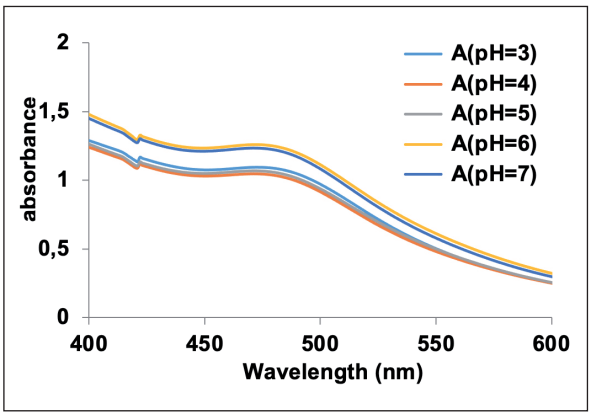

Fig. 2. UV-Vis absorption spectroscopy GNPs in different pH.

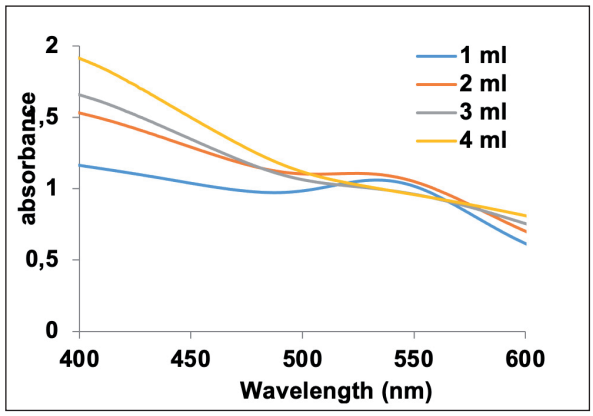

Fig. 3. UV-Vis absorption spectroscopy GNPs in various volumes of extract.

2 ml extract was added to each tube. The maximum absorption is related to gold salt with 3 mM concentration (Figure 4).

Results of optimum temperature

Finally, for studying optimum temperature, solutions with mentioned optimum conditions were prepared and were studied at different temperatures (25, 35, 55, 75°C). Absorption spectrum of solutions showed no remarkable difference in these temperatures but at 55°C there was higher adsorption spectrum. So, 55°C was the optimum temperature (Figure 5).

Characterization of the Au NPs by XRD and TEM

TEM was used to measure the size of synthesized NPs and to investigate their shape. The results con-

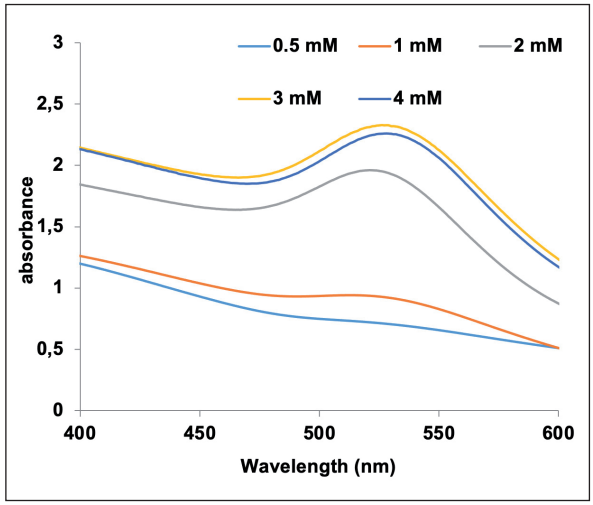

Fig. 4. UV-Vis absorption spectroscopy GNPs in various gold salt concentration.

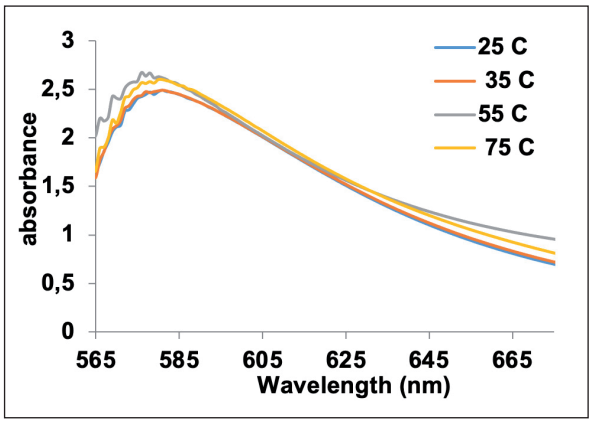

Fig. 5. UV-Vis absorption spectroscopy GNPs at various temperatures.

firmed that spherical and triangular gold NPs with the size of between 10-50 nm were synthesized (Figure 6a).

Furthermore, in order to identify the phase of crystalline NPs and to provide information on unit cell dimensions, XRD was applied (Figure 6b). The average size of crystalline particles was determined by measuring the width of the peaks formed in the samples using the Debye- Scherrer equation:

Equation 1: $D = 0.9\lambda / \beta \cos \theta$

Where β is the peak width at half the maximum height, λ, the wavelength of the X-rays is 1.4 nm, θ is the angle between the radiation beam and reflection, and D is the size of the crystalline particles. In areas of 2θ=38.21, 44.51, 64.90, 77.90, GNPs show sharp peaks, demonstrating the successful synthesis of NPs. The structural analysis shows that GNPs have a crystalline structure with Miller indexes (111), (200), (220) and (311) in a cubic network. The presence of sharp peaks in patterns shows a high degree of crystallinity for NPs.

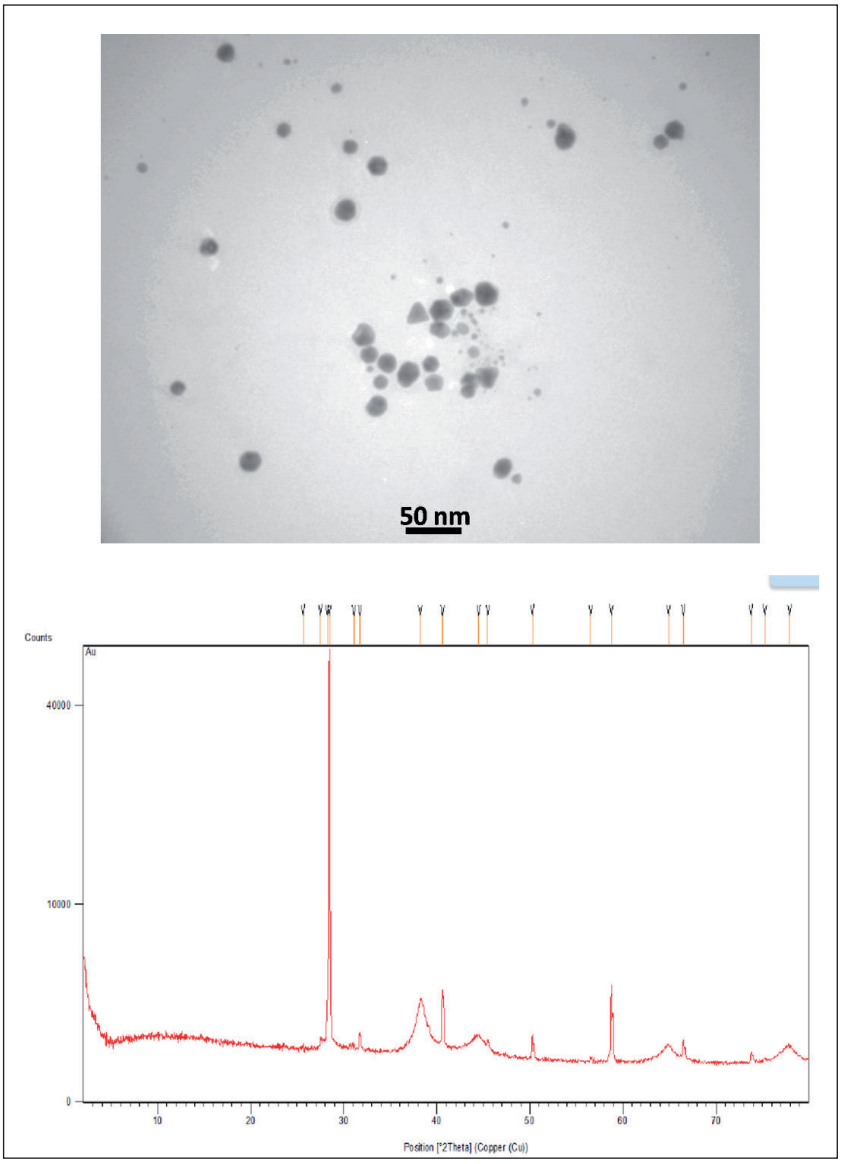

Fig. 6. *A*, The TEM picture of synthesized GNPs by walnut green shell. *B*, The XRD picture of synthesized GNPs by walnut green shell.

Results of the cell viability assay

The changes in cell viability of MCF7 treated with different doses of GNPs at 24h were determined using MTT method. Statistical analysis also found a significant difference among various GNPs doses on decreased cell viability of MCF7 cells in 24 h in dose-dependent fashion ($p < 0.05$) (Figure 7). The 50% inhibition (IC50) value evaluated after 24 h of GNPs against Michigan Cancer Foundation-7 (MCF7) cells was 3 mM ($p \leq 0.05$).

DISCUSSION

Much attention has been paid to gold nanoparticles because of the biocompatibility, better optical properties, and their capability to chemically modify their surface via addition of several kinds of ligands [14]. In this study, we used walnut green shell to synthesize gold NPs and characterized their anticancer, antioxidant properties using MTT assay and applied these NPs for cancer treatment. Marshall et al[8] recorded gold NPs accumulation into Brassica Juncea with diameters of 5-50 nm, as displayed via transmission electron microscopy (TEM), at 1120 and 760 ppm Au concentrations. The gold nanoparticles ability for biosynthesizing the flower of pharmacologically significant tree Couroupita guianensis has been shown by Geetha et al[9]. The synthesis process, which is one-step, fast and cost-effective, has been obtained. The gold nanoparticles biological synthesis has been reported by Sujitha and Kannan [10] through the HAuCl4 decline via applying the extract of citrus fruit juice as the stabilizing and decreasing agent. In this research, a different size and shape for the nanoparticles of gold were built whenever the reactants ratio was changed regarding chloroauric acid 1.0 mM solution. GNPs have been prepared by Elia et al[11] applying four different plants (Punica granatum, Lippia citriodora, Salvia officinalis, and Pelargonium graveolens) as stabilizing and decreasing agents. By applying three various approaches the GNPs size distributions were measured: nanoparticle-tracking analysis, the images of scanning electron microscopy analysis, and the dynamic light scattering. Two novel glycosides of α-tetralonyl were isolated by Wang et al[19] from the Juglans mandshurica green walnut husks. The entire compounds structural characterization was carried out via spectroscopic analysis, containing HR-ESI-MS, 2D NMR and 1D experiment. The isolated compounds were assayed for their cytotoxicity against two lines of human cancer cell, HeLa and A549. Inhibitory influences were displayed by four compounds[7-10] against two lines of human cancer cell with the values of GI50 between 5.8 and 1.3 μM[19]. Proteins from Juglans regia were isolated by Carrillo et al[20] and were hydrolyzed with various enzymes. A high anti-proliferative activity was indicated by the proteins of prolamin and Glutelin against the K-562 (leukemia) and PC-3 (prostate) cancer cells with 84.4 and 43.9 μg/mL values, respectively. The observed influence of highest inhibition, at 0.25 μg/mL concentration, was 50% in gastrointestinal digestion with Corolase PP and pepsin in a dose dependent manner against UACC62 (melanoma) cancer cells. Inhibitory impacts were indicated by pepsin hydrolysate against UACC62 cancer cells cancer at concentration of 71.0 μg/mL.

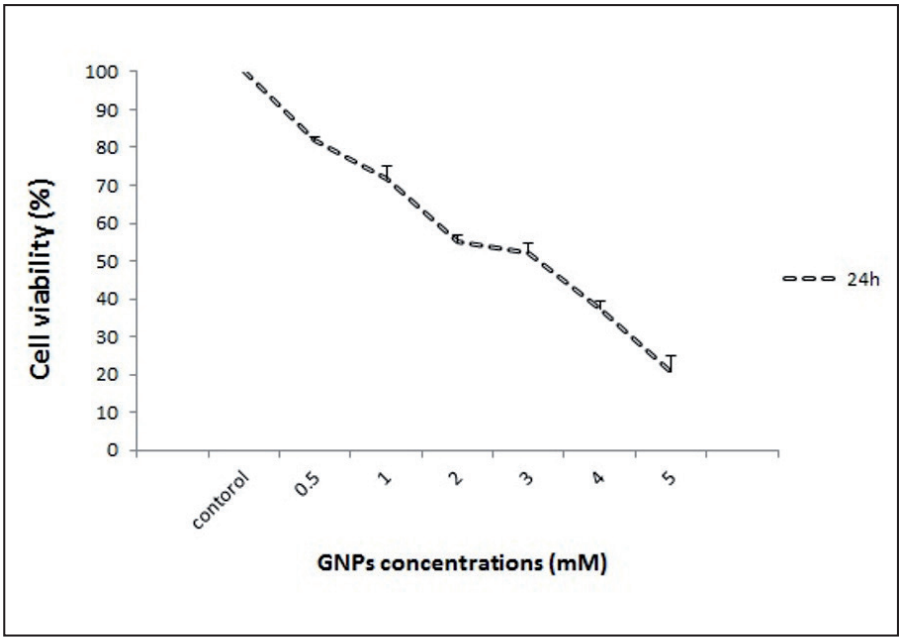

Fig. 7. The changes in viability of MCF7 cell lines treated with various doses of GNPs after 24 h, by MTT assay (<0.05).

In a dose-dependent manner, the influences were discussed. Neutrase enzyme inhibitory impacts were presented by the attained hydrolysate against UACC62 (melanoma) cancer cell at concentration of 25 µg/mL. The cytotoxicity was not indicated, neither by protein hydrolysates nor by proteins against VERO assay with normal cell (epithelial) [20]. Wang et al[19] improved a hybrid nanocomposite which is a new kind of thermal-fluorescent core-shell that indicates whether the composite has the effectiveness of cancer therapy in *in vivo* and *in vitro* assays and also has remarkable bio-compatibility[21]. A one-step and green technique was reported by Liang et al[22] for synthesizing the gold fluorescent nanoclusters via applying a cyclic commercialized acid peptide, arginine-glycine-aspartic as the template that can be used as fluorescent for staining the αvβ3 integrin-positive cancer cells, as well as radio sensitizing agents for increasing the effectiveness of killing radiation therapy[22]. The nanostructures of core-shell Fe3O4/Au were constructed by Izadian et al[23] applying a progressive synthesis approach which has two-steps, from the green husk extract of Juglans regia (walnut). This compound is a good candidate for further biomedical applications and cancer treatment[23]. Research studies show pH is one of the important parameters affecting NPs synthesis [24]. This effect is mostly on NPs sizes, not their shapes[25]. In addition, studies show in acidic pH, larger sizes (25-85 nm) of NPs and, in contrast, in alkali pH smaller sizes (5-20 nm), are made[26]. In NPs biosynthesis methods by plants, essence plays a reductant and stabilizing role[27].

CONCLUSIONS

In this study, a green method was used to synthesize Au NPs from *J. regia* green husk and the formation of Au structure was studied by TEM and XRD. Since many factors are effective in NPs synthesis, some of them studied in this paper show that at high volume, larger NPs probably lead to absorption reduction (Figure 3). Furthermore, absorption increases by increasing gold solution concentration that is due to rapid reactions between gold solution and extract. But, by exceeding gold optimum concentration a little reduction is seen in absorption that can probably be attributed to NPs sizes. This study reveals walnut's high potential in synthesis of metal gold NPs and its effects on cancer cells and there is an important development in the antioxidant and cytotoxicity characteristics of gold nanoparticles which are green synthesized. Statistical analysis also found a significant difference among various GNPs doses on decreased cell viability of MCF7 cell lines in 24 h in concentration-dependent fashion. The IC50 value evaluated after 24 h of GNPs against MCF7 cells caused 52% cell death at the concentration of 3 mM. Consequently, in addition to its many nutritional values, walnut can be used in medicine and pharmacology research.

Acknowledgments

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Conflict of Interest:

The authors declare no conflict of interest

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