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Biosynthesis of Silver Nanoparticles Using Hydroethanolic Extract of Cucurbita pepo L. Fruit and Their Anti-proliferative and Apoptotic Activity Against Breast Cancer Cell Line (MCF-7)

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Introduction: Cancer is the second leading cause of death all over the world and breast cancer is the second common member of cancers worldwide. In this study, silver nanoparticles (Ag-NPs) were synthesized; using hydroethanolic extract of Cucurbita pepo L. fruit and evaluated for their antiproliferative and apoptotic activities against MCF-7 cell line.
Methods: Ag-NPs formation was characterized by ultraviolet-visible spectroscopy, energy dispersive spectroscopy (EDX), scanning electron microscopy (SEM), dynamic light scattering (DLS), and Fourier transform infrared (FTIR) spectroscopy. Cytotoxicity and apoptosis responses were evaluated by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and dual acridine orange/ethidium bromide (AO/EB) fluorescent staining, respectively.
Results: Our results demonstrated the formation of Ag-NPs by Cucurbita pepo L. fruit extract discoloration to dark black. This transformation revealed their slightly aggregated shapes to quasi-spherical form with a mean diameter of 104 nm. The zeta potential value was -42.3 mV for any Ag-NPs. These results indicated the successful formation of Ag-NPs for cellular uptake. MTT results showed that Ag-NPs significantly decreased the viability and induced MCF-7 cells apoptosis in a dose-dependent manner; especially at concentrations of 50 and 250 μg/mL.
Conclusions: In conclusion, according to the results of the present study, biologically synthesized Ag-NPs induce apoptotic and cytotoxic effects against breast cancer cell lines in a dose-dependent manner.

INTRODUCTION

Cancer is an increasingly growing malfunction in the human population and is known as one of the hazardous problems for human health and life. In addition, it is a big challenge for health researchers to control and cure the disease. Vegetables and fruits, commonly consumed by humans, help to lower cancer frequency in the population [1]. Pumpkin or Cucurbita pepo L. (F: Cucurbitaceae) is an annual, single-sex plant with bright yellow flowers. Features of pumpkin bushes include creeping stems with approximately 10 mm diameter lying on the soil surface, coarse hair, oval and broad leaves with 15 to 30 cm diameter at the polar base, and various shapes at sharp edges covered by coarse hairs [2]. China, India, Ukraine, Egypt, and the United States are the largest producers of this plant, and Iran is the fifth one [3, 4]. Pumpkin fruit contains active biological compounds such as polysaccharides, para-aminobenzoic acids, oils, sterols, proteins, and peptides, being a good source of carotenoids and gamma-aminobutyric acid [5]. Researchers have focused on this plant for decades because of its background in the traditional medicine of different societies. This plant has anti-diabetic, anti-hypertensive, anti-tumor, immunomodulatory, antibacterial, anti-cholesterol, anti-inflammatory, and anti-fever effects [6, 7]. Diets containing pumpkin seeds are associated with a lower risk of lung, breast, gastrointestinal, and colon cancers [8]. Carotenoids in pumpkin seeds and fruit are involved in inhibiting prostate cancer [9, 10]. The pumpkin fruit extract has been reported to reduce tumor weight in S-180 mice [11].

Nanotechnology is a newly growing technology for manufacturing new nanoscale materials with many applications [12]. The size of materials used by nanoscience and nanotechnology are typically in the range of 0.2 to 100 nanometers and their properties change as they approach nanoscales [13]. Nanomaterials are used in a wide range of applications such as bio-engineering, biosensors, cosmetics, nanoparticles, catalysts, drug delivery, medicine, etc. [14]. Significant progress has been made in the application of nanotechnology and new methods of preparing and using nanomaterials. Recently, some growing interest has been observed in the synthesis and properties of noble metal nanoparticles such as gold, silver, and platinum in the field of nanomedicine [15]. Silver nanoparticles (Ag-NPs) are extensively used because of their novel properties and encouraging applications as anticancer and antimicrobial agents [16-18]. Concerns about environmental pollution and subsequent hazardous side-effects increased following the advent of newly increasing methods for the chemical synthesis of nanoparticles. Therefore, it is crucial to use certain methods based on green chemistry as they are non-toxic and eco-friendly passages for the environment [19]. Numerous methods of nanoparticle synthesis (chemical, physical, and green) are being developed and used to synthesize various types of nanoparticles. However, the green synthesis technique is used as an economical and nature-friendly method and involves natural reducing agents and stabilizers for the formation of nanoparticles [20, 21]. Plants and derivatives of their extracts are widely used to manufacture nanoparticles [21]. The plant extract possesses different biomolecules with carboxylic and hydroxyl groups that can act as reducing and stabilizers agents. Not only plant leaf, but also other plant segments such as roots, bark, fruit, fruit peels, and callus have been applied in different shapes and sizes for the synthesis of metal NPs such as Au, Pt, Ag, etc. [22]. Different studies showed that Ag-NPs were synthesized with different herbal extracts, such as Justica wynaadensis leaves extract [23], Camellia Sinensis (L.) extract [24], Syzygium guineense (Willd.) leaf extract [25], and Sansevieria Roxburghiana Schult. & Schult. extract [26]. Since there is poor knowledge about the synthesis of green nanoparticles using ethanolic extracts of pumpkin fruit, the present study contributes to medical and pharmaceutical researches by synthesis of green pumpkin NPs with their specific properties. This study aims to add Ag-NPs based bio-reduction process; using hydroethanolic extract of C. pepo fruit, characterize the biologically synthesized Ag-NPs separately, and evaluate their potentials for exerting their apoptotic and anti-cancer effects on MCF-7 human breast cancer cells and human umbilical vein endothelial cells (HUVECs) as the normal cells.

METHODS

Preparation of Pumpkin Fruit Extract

Pumpkin fruit was bought from a local fruit market. The skin and seeds were separated and the fruit was chopped into thin slices and dried in the shade. Dried slices of pumpkin fruit were ground and then, 10 gr of the powder was added to 100 mL of 70% ethanol and was shaken for 72 h. Then, the sample was centrifuged and the supernatant was separated and passed through Watman's paper. It was subsequently transferred to a rotary device to separate the alcohol phase from the water phase.

Synthesis of Nano-particles

About 30 cc of pumpkin fruit extract was added to 70 cc of 1 mM silver nitrate solution drop by drop. The solution was shaken under the dark condition at room temperature for 24 h to change its color from yellow to grayish black. In addition, the solution absorbance was examined in the range of 300-700 nm; using a spectrophotometer. Subsequently, a solution containing nanoparticles was centrifuged at 12000 rpm for 15 min, then, washed twice with distilled water, and once with alcohol, the supernatant was discarded and the sample was dried.

Characterization of Green Synthesized Ag-NPs

The morphology of Ag-NPs was evaluated; using scanning electron microscopy (SEM). DLS was used to determine the hydrodynamic diameter of Ag-NPs and their size distribution. Zetasizer device was used to determine the sizes and surface loads of Ag-NPs. Elemental analysis was operated; using energy-dispersive X-ray differentiation (EDX) to investigate the purity and approximate amount of each element for verifying the synthesis of Ag-NPs. Also, the major active bio-compounds in pumpkin fruit extract and Ag-NPs were investigated and evaluated; using Fourier transform infrared spectroscopy (FT-IR) with KBr pellets in the range of 4000–400 cm^{-1}.

Cell Culture

Cells used in this study were from MCF-7 cell line (a type of breast cancer cells), and HUVECs as normal cells that were purchased from Pasteur Institute of Iran. They were cultured on the Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Proliferated cells were frozen for later use.

Cell Cytotoxicity of Ag-NPs Coated With Pumpkin Fruit Extract and Hydroethanolic Extract of Cucurbita pepo L. Fruit

To perform a cytotoxicity assay, MCF-7 and HUVECs were seeded on a 96-well cell culture plate at a density of 1×10^4 cells per well. The plates were transferred to an incubator with 95% relative humidity and 5% CO2 at 37ºC. After 24 h, Ag-NPs and hydroethanolic extract of C. pepo fruit with different concentrations (2, 10, 50, and 250 mg/mL) were cultured on DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin. The group not receiving any supplements was considered as the control group. After 24 h of incubation with Ag-NPs or hydroethanolic extract of C. pepo fruit, about 20 μL of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution at a concentration of 5 mg/mL Dulbecco's phosphate-buffered saline (DPBS) was added to each well and incubated in 37ºC for 4 h. At the end of the incubation period, the medium was removed and 100 μL of Dimethylsulfoxide (DMSO) was added to each well. Reading was performed at 570 nm with an ELISA reader.

Investigation of Apoptosis by Acridine Orange-Ethidium Bromide Dye

For this purpose, cells were seeded on a 24-well plate and treated with different concentrations of Ag-NPs and hydroethanolic extract of pumpkin fruit. Supernatants were removed after 24 hours. Each well was washed with DPBS solution and removed later. Then, paraformaldehyde solution was added to it and maintained for 15-20 min. The fixing solution was removed and re-washed with DPBS. Finally, ethidium bromide and acridine orange (1:1) dye were added to each well under dark conditions. Cells were photographed and evaluated under a fluorescent microscope.

Statistical Analysis

Duncan test and ANOVA were performed to examine differences between groups. Values are expressed as mean±SD of the ۳ replicates per experiment.

RESULTS

Hydroethanolic extract of C. pepo fruit was used to synthesis Ag-NPs under facile conditions. After reacting for 24 h, pumpkin fruit extract turned to grayish-black; indicating the formation of Ag-NPs (Figure 1).

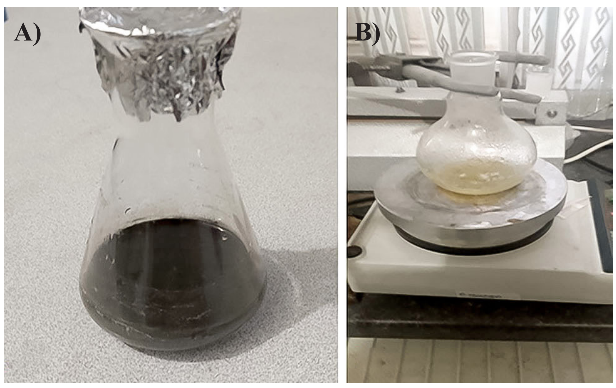

Figure 1: Color Change of Reaction Medium
The color of the reaction medium is gradually changed to dark after 24 hours. A) Erlenmeyer contain grayish-black colored Ag-nanoparticles solution; B) Balloon contain pumpkin fruit extract

Measurements of UV-visible spectra proved direct bio-reduction of Ag^+ to Ag^0 and formation of Ag-NPs. A peak at 430 nm present on UV-vis spectra of Ag-NPs represents the complete synthesis of Ag-NPs (Figure 2).

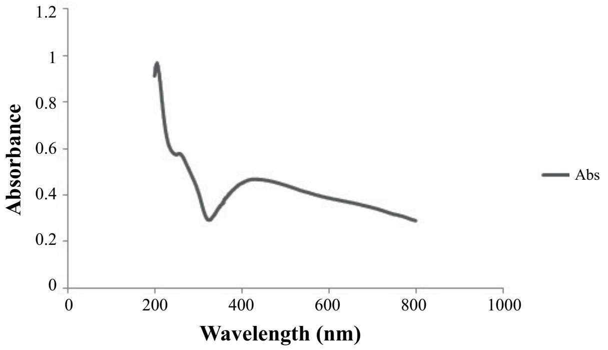

Figure 2: UV-visible Absorbance Spectrum of Ag-nanoparticles Ag-nanoparticles producing a peak at 430 nm

Energy-dispersive X-ray (EDX) was employed to analyze the chemically elemental composition of Ag-NPs (Figure 3). Five signals can be observed from EDX analysis: a strong signal from Ag atom (79.63%) along with those from C atom (8.8%), O atom (2.73%), N atom (4.01%), and Cl atom (4.83%). No visible peaks were observed for other elements or impurities.

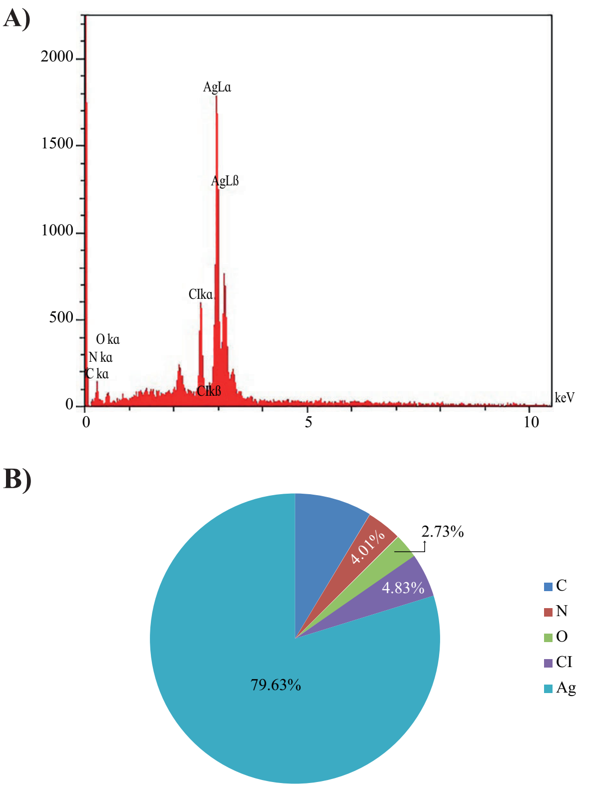

Figure 3: EDX Spectra of Green Synthetic Ag-nanoparticles

The FTIR spectra of hydroethanolic extract of C. pepo fruit and Ag-NPs synthesized; using the fruit extracts, are shown in Figure 4. Figure 4A indicates four centered main peaks at 3417, 1638, 1387, and 1111 cm^{-1} and nine weak peaks 3920, 3888, 3871, 3856, 3839, 3824, 3788, 3729, and 3665 cm^{-1}. The widest observed peak at a wavenumber of 2900–3600 cm^{-1} was related to the stretching vibrations of hydroxyl groups (O-H) which had a key role in the reduction of Ag ions to their elements and formation of the Ag-NPs. The band at 1638 cm^{-1} ascribed to the stretching mode of amide C═O. Furthermore, a minor centered peak at 1387 cm^{-1} could be related to the existence of carboxylic components. Also, absorbance bands of hydroethanolic extract of pumpkin fruit were observed at 1111 cm^{-1} assigned to C–N amines stretch (Figure 4A). Acting as capping agents and reducing, biomolecules may be responsible for Ag-NPs stabilization. P-Ag-NPs were evaluated for absorption spikes which were determined at 3414, 1637, 1384, 1075, 617, and 481 cm^{-1} (Figures 4B).

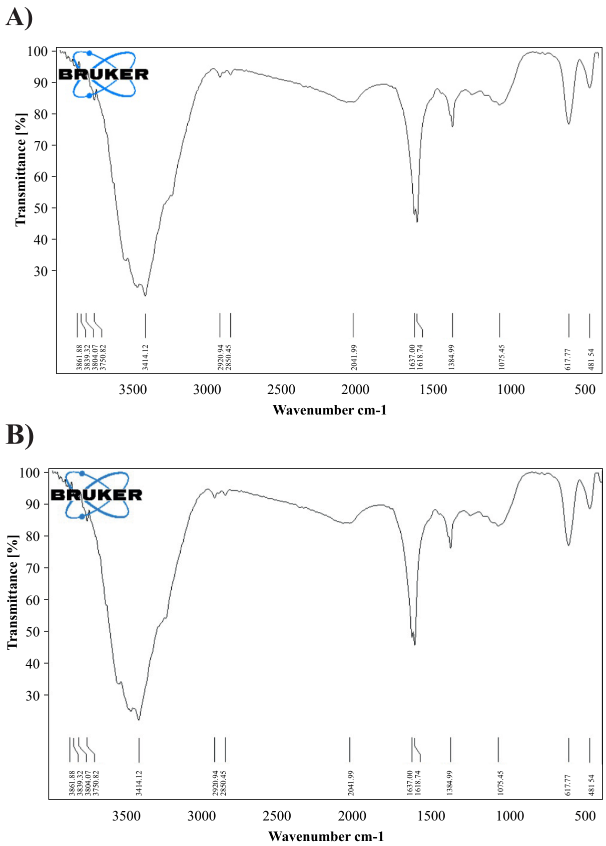

Figure 4: FTIR Spectrums of Hydroethanolic Extract of Cucurbita pepo L. Fruit and Ag-nanoparticles Synthesized From Pumpkin Fruit Extract

A) Ag-nanoparticles; B) synthesized from pumpkin fruit extract

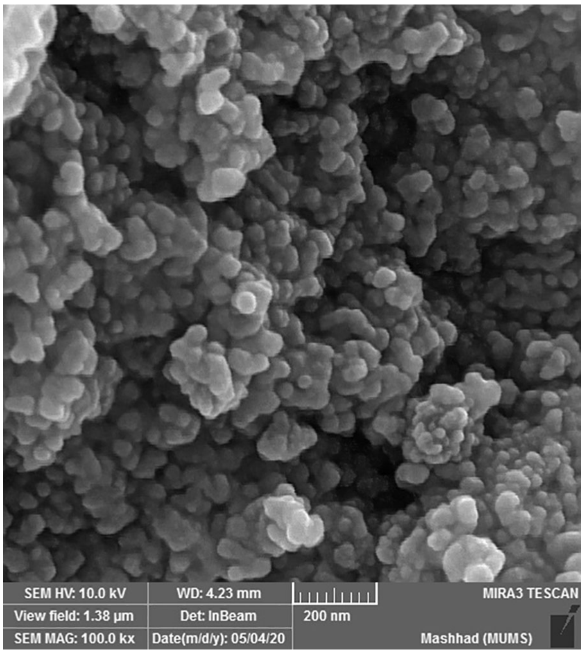

Figure 5: FE-SEM Image of Green Synthetic Ag-nanoparticles

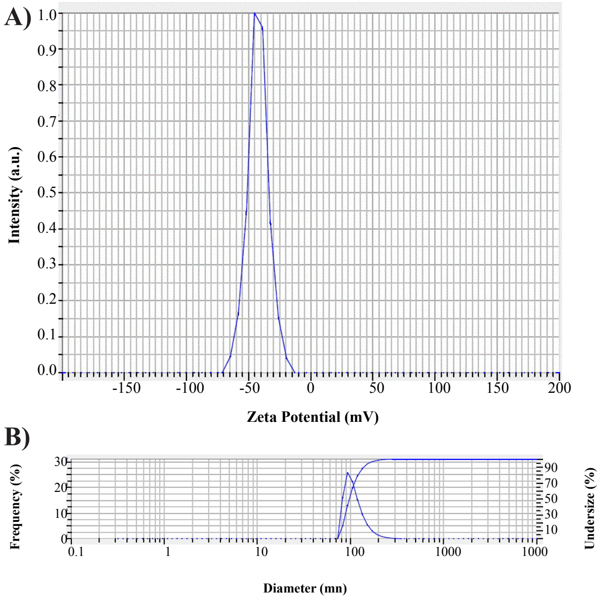

Figure 6: Characterization of Biosynthesized Ag-nanoparticles (Ag-NPs)
A) dynamic light scattering; B) zeta potential

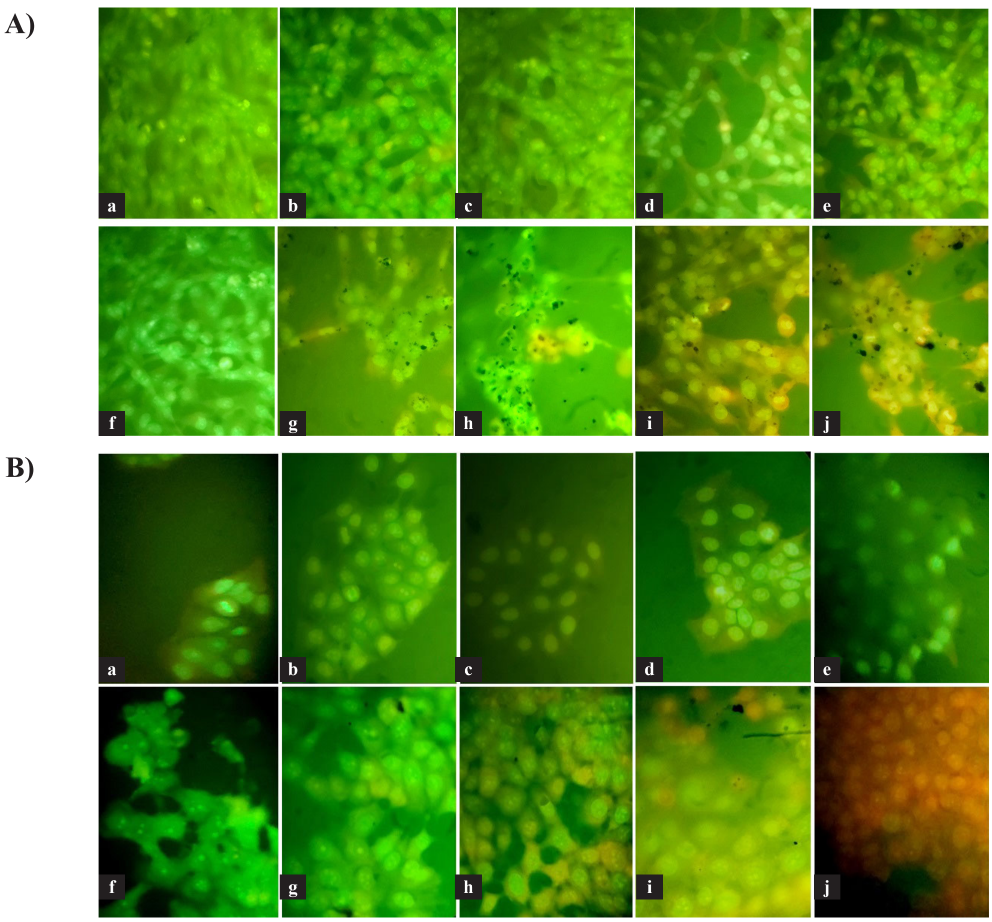

Figure 7: Apoptotic Detection: Acridine Orange-Ethidium Bromide Staining (AO/EtBr) in HUVEC Cells
A) HUVEC cells. (a and f) control (untreated-HUVEC cells), (b) 2, (c) 10, (d) 50 and (e) 250 µg/mL of hydroethanolic extract of Cucurbita pepo L. fruit for 24 h. (g) 2, (h) 10, (i) 50 and (j) 250 µg/mL of Ag-NPs for 24 h; B) MCF-7 cells. (a and f) control (untreated-MCF-7 cells), (b) 2, (c) 10, (d) 50 and (e) 250 µg/mL of hydroethanolic extract of Cucurbita pepo L. fruit for 24 h. (g) 2, (h) 10, (i) 50 and (j) 250 µg/mL of Ag-NPs for 24 h.

As shown by SEM images, Ag-NPs are relatively uniform and quasi-spherical (Figure 5). Particle size is found to be around 104 nm. The value of the corresponding zeta potential is -42.3 mV as shown in Figure 6.

Results obtained from the induction of apoptosis; using dual acridine orange/ethidium bromide (AO/EB) fluorescent staining, are summarized in Figure 7. Data on dual fluorescent staining clearly showed that green synthesized Ag-NPs induced the cell death of MCF-7 cells.

Growth inhibition of MCF-7 and HUVEC cells by different concentrations of green synthetic Ag-NPs and hydroethanolic extract of C. pepo fruit was evaluated by MTT assay (Table 1). The addition of hydroethanolic extract of C. pepo fruit to normal cells culture medium resulted in a significant rise in cell viability in comparison to the control group ($P<0.05$). In addition, the percentage of viable MCF-7 cells was significantly decreased in groups that received the hydroethanolic extract of Cucurbita pepo L. fruit (10, 50, and 250 µg/mL) in comparison with the control group and the group receiving 2 µg/mL of Cucurbita pepo L. fruit ($P<0.05$). Cell viability in both cancer and normal cells was significantly reduced in the treated group that received Ag-NPs in a dose-dependent manner in comparison with the control group ($P<0.05$). The highest rate of cytotoxicity was observed at higher concentrations of Ag-NPs. However, the toxicity of Ag-NPs on cancer cells was higher than normal cells (Table 1).

The morphological assay of cell death was investigated; using AO/EB dye staining to evaluate the apoptotic effects of Ag-NPs and hydroethanolic extract of C. pepo fruit on HUVEC and MCF-7 cells (Figure 7). The cytotoxic efficacy of Ag-NPs in inducing early and late apoptosis was studied in a dose-dependent manner. As the concentration of Ag-NPs exposure increased, orange-stained cells which showed high levels of apoptotic cell death became more than the bright green nucleus.

DISCUSSION

Ag-NPs have been used extensively in the fields of drug delivery, cancer treatment, anti-microbial studies, nanotechnology, biotechnology, and biomedicine [27]. Different methods are applicable for synthesizing nanoparticles. However, most methods use hazardous materials necessary to produce nanoparticles. Other limitations of these approaches are the low production of nanoparticles, energy-intensive diversion processes, and defective purification systems [28]. Accordingly, over the past few decades, nanoparticle synthesis strategies have concentrated on the development of the eco-friendly and rapid green approach to cost-effective synthesis methods [29-33]. Extracts of many natural plants with a rich source of functional molecules have been verified as the capping and reducing agents for the synthesis of Ag-NPs [34]. This survey investigates the biosynthesis of C. pepo silver nanoparticles and evaluates the anti-cancer potentials of nanoparticles; using the hydroethanolic fruit extract of C. pepo. In exposure to the plant extracts, reduction of silver ion Ag^+ to silver particles Ag^0 is followed by color changes. Reduction of Ag^+ to Ag^0 nanoparticles results in the solution color changes from pale yellow to grayish-black after 24 h. An absorbance peak of about 430 nm indicates that Ag-NPs are found in the solution because surface plasmon

Table 1: Evaluation of the Cell Viability by the MTT Colorimetric Method of Different Concentrations of Green Synthetic Ag-NPs and Hydroethanolic Extract of Cucurbita pepo L. Fruit [a]

	MCF-7 Cells, mean±SD	HUVEC Cells, mean±SD
Control	100	100
Control +2 µg/ml (hydroethanolic extract of Cucurbita pepo L. fruit)	103.39±1.72	105.91±4.88
Control +10 µg/ml (hydroethanolic extract of Cucurbita pepo L. fruit)	95.43±2.43	105.55±2.52
Control +50 µg/ml (hydroethanolic extract of Cucurbita pepo L. fruit)	93.26±2.72	106.69±2.90
Control +250 µg/ml (hydroethanolic extract of Cucurbita pepo L. fruit)	93.63±1.79	121.45±5.08
Control +2 µg/ml Ag-NPs	88.58±1.72	93.75±0.77
Control +10 µg/ml Ag-NPs	72.65±2.18	85.35±4.71
Control +50 µg/ml Ag-NPs	34.67±3.74	70.46±4.10
Control +250 µg/ml Ag-NPs	17.71±1.19	38.17±3.51

[a] Significant differences ($P<0.05$)

resonance (SPR) electrons exist on the surface of nanoparticles [35]. A very strong peak at 3414 cm^{-1} has been indicated by the FTIR spectrum of Ag-NPs in Figure 4 which is assigned as −OH stretching in alcohols and phenolic compounds [36]. C=C aromatic vibrations are assigned a medium intense band at 1637 cm^{-1} [37]. Oxidation of NH2 group of amines and reduction of Ag^+ (silver cation) to Ag^0 (metal silver) from a peak at 1384 cm^{-1} related to the stretching vibration of the nitro group N=O [38]. The presence of C–O stretching of alcohols, amide, ester, and ether groups has been evidenced by the peak at 1075 cm^{-1} [39]. Peaks at 481 cm^{-1} and 617 cm^{-1} correspond to aliphatic iodine compounds, C–I stretch alcohol, and OH out-of-plane bending [40].

EDX spectra of synthesized Ag-NPs; using hydroethanolic extracts of C. pepo fruit as a precursor are given in Figure 3. The surface of nanoparticles which contains positive or negative charges provides stability and prevents nanoparticles from aggregating by pushing the same charges [41]. Green synthetic Ag-NPs show zeta potential values of -42.3 mV. Results from negative values strongly confirmed repulsion among particles which leads to increased stability of nanoparticles [42]. Quasi-spherical shape and the low number of agglomerated Ag-NPs are demonstrated by SEM image. The average size of particles is estimated at 104 nm. Using Glycyrrhiza glabra root extracts, in their study, Dinesh et al., [43] found 20-30 nm as nanoparticles sizes. Using Azadirachta indica A. Juss. leaf extracts, Renugadevi and Aswini [44] reported sizes between 41 and 130 nm based on SEM images. Cytotoxicity was examined by some studies performed on green synthetic Ag-NPs used against MCF-7 breast cancer cells. Ag-NPs are a sort of drug functionalized by hydroethanolic extracts from C. pepo fruit. Similar to our technique for Ag-NPs synthesis, synthesizing Ag-NPs by using aqueous leaf extracts from Cucumis prophetarum L. exhibited anti-proliferative effects on different cancer cells including MDA-MB-231, HepG2, AS49, and MCF-7 [45]. Green Ag-NPs synthesized by Nepeta deflersiana Schweinf. extracts induced concentration-dependent cytotoxicity in human cervical cancer cells and significantly increased the reactive oxygen species (ROS) amounts and lipid peroxidation, and reduced mitochondrial membrane potential and glutathione concentrations [46]. Anti-cancer activity of green synthetic Ag-NPs is confirmed by different cancer cell lines; for example, Ag-NPs synthesized using Artemisia turcomanica Gand leaf extracts with an average size of 22 nm affecting gastric cancer cell line (AGS) [39], Ag-NPs synthesized by Cynara scolymus L. leaf extracts with an average size of 98.47 nm affecting MCF-7 cells [27], Ag-NPs synthesized by Seaweed Gelidiella sp. whole seaweed extracts with an average size of 31.25 nm affecting Hep-2 cells [47], Ag-NPs synthesized by Brassica oleracea Caulifower florets extracts with an average size of 48 nm affecting MCF-7 cells [48], and Ag-NPs synthesized by Cissus quadrangularis L.stem extracts with an average size of 25-56 nm affecting Hep-2 cells [49]. Similar to our results, the results from different studies confirmed apoptotic effects of green synthetic Ag-NPs on different cancer cell lines [38, 50, 51].

In this study, we tried to introduce a simple method, and in the shortest possible time to produce Ag-NPs use hydro-ethanolic extracts of C. pepo fruit as a capping and reducing agent. This synthesis method is faster, cheaper (with minimal sophistication), and environment-friendly, representing a treatment option with high accessibility, the least harmful side effects, and higher economic profits. Ag-NPs affected MCF-7 and HUVEC cell lines showed a decrease in cell viability as the concentration increased but this reduction in viability was greater for cancer cells. More detailed research is required to use the Ag-NPs in experimental animals to analyze their effect in an in vivo system or even in other cell lines.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ETHICS APPROVAL

The Ethical approval of the study was obtained from the Ethics Committee of the Razi University (IR. RAZI.REC.1399.031).

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